THE USE OF METABOLITES IN THE RESTORATION OF THE VIABILITY OF HEAT AND CHEMICALLY INACTIVATED ESCHERICHIA COLI[1]

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On the basis of accepted methods for testing sterility, organisms failing to grow in a complete growth medium are considered to be nonviable. It has been observed, however, that bacterial suspensions sterilized by the action of ultraviolet irradiation can be rendered viable when incubated with suitable metabolites (Heinmets, 1953; Heinmets *et al.*, 1954). This suggested that the concept of the "killed" state is relative. In current view the "killed" state would represent only a loss of cellular viability in terms of inability to multiply, but not necessarily in the termination of synthetic and metabolic activity. It appears that the loss of a particular cellular function need not produce the total loss of viability. To the contrary, it may be assumed that when the more basic functional activities of the cellular processes are left essentially intact, the cells may regain viability if "guided" to function specifically to reestablish the specific synthetic capacity necessary for cellular division. This concept presupposes that the synthetic and metabolic processes are more basic in cellular activity than the process of cellular division. Therefore, being only a derivative, fission results from a properly organized and balanced action of basic functional processes. This view is supported by the accepted fact that microorganisms which have lost their ability to divide are still capable of growth. The result is a formation of filamentous variants. Such loss of ability to multiply may be caused by many factors such as: irradiation injury, deficiency of the growth medium, or the presence of certain inhibitors in the medium. All of the aforementioned factors can distort or unbalance the synthetic and metabolic processes of the cell, thus resulting in the impairment of division mechanism.

[1] This work is not to be construed as necessarily reflecting the views of the Department of the Navy.

We have analyzed previously the character of cellular injury produced by ultraviolet irradiation, and in view of experimental evidence a multiple pattern of injury is indicated (Heinmets, 1953). The fact that bacteria can recover from irradiation injury which involves large numbers of structural elements suggested the possibility that when loss of viability is produced by other modes of cellular injury, restoration could take place when suitable conditions are made available. As a result of these considerations, it was decided to investigate the phenomenon of restoration of viability in *Escherichia coli*, strain B/r, when "killed" by the action of heat and chemicals.

EXPERIMENTAL METHODS

Escherichia coli, strain B/r, was used in all experiments. Cultures were grown for 18 hours under constant aeration at 37 C in M-9 medium (Anderson, 1946). The test organisms were removed from the liquid culture media by centrifugation. They were washed twice with distilled water and resuspended in 0.1 M phosphate buffer (pH 7.0). Buffer was composed of KH_2PO_4 and Na_2HPO_4. The number of viable cells in untreated and treated suspensions was determined by plating the bacteria on M-9 agar and incubating for 48 hours at 37 C.

Methods of inactivation. (1) *Heat.* Phosphate buffer, pH 7, was heated to the desired temperature, and one ml of the standard bacterial suspension was added to 99 ml of hot buffer. The mixture was agitated continuously by a magnetic stirrer. Samples were taken at desired time intervals and immediately diluted in a 1:1 ratio with cold buffer. (2) *Chlorine.* To the 99 ml of phosphate buffer which contained one ppm free chlorine was added one ml of the standard bacterial suspension. The mixture was agitated continuously by a magnetic stirrer. Samples

were removed at desired time intervals, and in order to stop chlorine action the suspension was diluted in 1:1 ratio with a buffer which contained 40 ppm of sodium thiosulfate. (3) *Hydrogen peroxide*, *"zephiran" chloride*, and *ethyl alcohol* also were used for inactivation, and similar procedures as described for chlorine were used. H_2O_2 was neutralized by catalase; and "zephiran" chloride, by a mixture of 3 per cent "tween-80" and 0.2 per cent asolectin. Ethyl alcohol was made ineffective by initial 1:100 dilution of the bacterial suspension with buffer, and the residue of alcohol was removed by dialysis at 5 C. Alcohol also can be removed by washing the bacteria twice by centrifugation.

Methods of reactivation. Bacteria in phosphate buffer (pH 7), after chemical or heat treatment, were diluted in 1:1 ratio with various metabolites (0.2 per cent) or substrates and were incubated for 24 hours at 37 C. Subsequently, all samples were plated out in the usual manner, and the number of viable cells was compared with that of the control suspensions.

RESULTS

Influence of contact time. The effect of the contact time of chlorine on bacterial viability is presented in table 1. It is evident that after one minute of contact with chlorine bacterial suspension did not contain viable cells. This, according to the conventional testing, is interpreted that no cells capable of growing in normal culture medium survived after such treatment. The increase of contact time should potentially increase the probability of total "kill". From a practical point of view, such bacterial suspensions are considered to be sterile. However, when such "sterile" suspensions are incubated for 24 hours in buffered pyruvate solution and subsequently plated, a large number of colonies will develop on M-9 agar plates. The sterile sample (chlorine contact time one minute) contains 2.9×10^5 viable organisms after pyruvate incubation, indicating that chlorine treated samples are "sterile" only when conventional plating procedures are used. Buffer incubation alone did not produce viable cells. Chlorine contact time in this experiment has no significant effect on metabolic recovery. It is likely that bacteria had interacted in a relatively short time with all available chlorine, and further contact time did not have a significant influence on viability.

TABLE 1

Chlorine inactivation and metabolic reactivation of Escherichia coli, strain B/r

Bacteria suspended in phosphate buffer at pH 7, which contains 1 ppm chlorine and samples removed at various time intervals and diluted 1:1 with a solution containing 40 ppm thiosulfate, part of the samples incubated at 37 C for 24 hours and plated.

ORDER NO.	CONTACT TIME WITH CHLORINE	PLATED IMMEDIATELY	INCUBATED IN	
			Pyruvate (0.1%) 24 hours 37 C	Buffer (pH 7)
	min			
1	Control	7.1×10^6	3.3×10^6	—
2	1	0	2.9×10^5	—
3	2	0	2.6×10^5	0
4	4	0	2.5×10^5	—
5	10	0	2.0×10^5	0
6	15	0	1.5×10^5	—

Table 2 summarizes the experiments concerning the heat contact time and subsequent metabolic reactivation. Since ultraviolet inactivation and reactivation experiments had indicated that many other intermediary metabolites besides pyruvate were capable of inducing cellular recovery from radiation injury, it was decided to test other metabolites closely related to pyruvate metabolism. Selection of test temperatures was motivated by the following reasoning. At 60 C, for a short period of time, protein denaturation and enzyme inactivation are relatively minor; the thermal injury at this level may be partially reversible. On the other hand, at 72 C a more drastic denaturation of proteins and inactivation of enzymes take place and extensive thermal injury is expected. The flash pasteurization method consists of heating liquids to 71.1 C for 15 seconds. Restoration of cellular viability after such treatment was considered highly improbable. The data in table 2 indicate that heat treatment at 60 C for 10 minutes is not sufficient to sterilize the suspension. However, 45 seconds at 72 C were sufficient to sterilize the suspension when plated immediately. Incubation in phosphate buffer at pH 7 did not produce any viable cells in the 45 second sample, whereas the organisms in the 15 second sample lost their viability during incubation. It appears that incubation after heat treatment is detrimental to the bacteria. However, when incubation takes place

TABLE 2

Heat inactivation and metabolic reactivation of Escherichia coli, strain B/r

One ml suspension of bacteria added to 100 ml phosphate buffer at 60 and 71 C; samples removed at suitable intervals and mixed with equal amount of cold buffer; part of the samples incubated at 37 C for 24 hours and plated.

NO.	TYPE OF SAMPLE	VIABLE CELL COUNT			
		1 min at 60 C	10 min at 60 C	15 sec at 72 C	45 sec at 72 C
1	Heated	9.3×10^3	8×10^1	1×10^1	0
2	Heated, incubated in buffer	7.2×10^2	7×10^1	0	0
	Incubated at 37 C for 24 hours with substrate				
3	Acetate	5.5×10^3	4.8×10^3	—	—
4	Oxalacetic acid	3.2×10^6	1.3×10^6	7.2×10^3	4.4×10^3
5	Pyruvate	1.1×10^5	6.0×10^1	1.0×10^2	1.0×10^2

Number of viable cells before heating: (1) Sample heated at 60 C—7.7×10^6; (2) Sample heated at 72 C—1.6×10^6.

in the presence of oxalacetic acid or pyruvate, a substantial increase of viable cells is noted. It is remarkable that bacterial suspension heated at 72 C for 45 seconds exceeding normal pasteurization limits still contains a number of viable cells after incubation with metabolites. Sodium acetate is capable of inducing only minor cellular recovery. It is interesting to note the difference between the organisms' recovery capacity in the presence of the metabolites in regard to heat sensitivity. One minute heat treatment at 60 C indicates a tenfold difference in pyruvate and oxalacetate recovery effect. However, 10 minutes at 60 C destroy all pyruvate recovery but only slightly affect oxalacetate and acetate recovery.

Influence of various metabolites and their combinations on restoration of viability of "killed" bacteria. The original observation that ultraviolet irradiated bacteria can be reactivated following pyruvate incubation (Heinmets, 1953) demonstrated the basic cellular potentiality to overcome, under controlled conditions, a specific mode of injury. It is obvious that in a multiple-pattern injury a broader spectrum of metabolites may induce a more complete cellular recovery. Furthermore, the character of cellular injury will vary, depending on the mode of action of a physical or chemical agent. The chemical mode of injury could be highly specific and could be restricted only to a certain structural element of the cell. Such considerations suggested studies on the reactivating action of various metabolites and their combinations on various modes of cellular injury. Present studies include only a limited number of compounds which are involved in the intermediary metabolism of the cell. In order to rule out multiplication when injured cells are incubated with metabolites which could act as growth stimulants (Johnson and Cohn, 1952), heat or chemically "sterilized" suspensions were plated on M-9 agar or inoculated into nutrient broth to check sterility. No viability could be detected by these methods. A presentation of the effectiveness of various metabolites on heat sterilized bacteria is given in table 3. The original suspension contained 3.1×10^6 viable cells which were heat treated for 30 seconds at 71 C. Incubation for 24 hours in nutrient broth or plating for 48 hours on M-9 agar plates indicated that no viable cells were present. However, incubation of such "sterile" suspensions for 24 hours with various buffered (pH 7) metabolites produces a large number of viable cells. The most active single metabolites are sodium citrate, oxalacetic acid, and lactic acid. A very extensive cellular recovery was achieved when all metabolites were mixed in equal proportions. It is indicated that about 70 per cent of "killed" bacteria have recovered and are capable of growing on M-9 plating medium. Spectroscopic studies indicate that there is no measurable difference in optical density values of bacterial suspensions before and after incubation with a metabolite. This confirms that the presence of viable cells is not due to cellular multiplication.

The observations on the reversal of heat in-

TABLE 3

Restoration of viability by the use of metabolites in Escherichia coli, strain B/r, which have been "killed" by chemicals and heat

SUBSTRATE NUMBER	MODE OF TREATMENT	NUMBER OF VIABLE CELLS				
		Heat	Chlorine	H_2O_2	"Zephiran" chloride	Ethyl alcohol
	Before treatment	3.1×10^6	1.1×10^6	1.4×10^7	3.5×10^6	3.5×10^6
	After treatment	0	0	8.0×10^1	0	0
	Substrate	Treated samples incubated with substrate at 37 C for 24 hours				
1	Buffer (pH 7)	0	0	0	0	3.1×10^3
2	Sodium pyruvate	0	0	8.0×10^1	0	4.4×10^4
3	Oxalacetic acid	2.3×10^3	3.2×10^3	3.0×10^3	1.4×10^3	2.2×10^4
4	Sodium acetate	0	0	1.6×10^3	0	3.6×10^4
5	Sodium citrate	*1.3×10^4*	*1.1×10^4*	*1.4×10^5*	*$>1.5 \times 10^6$*	4.1×10^1
6	*cis*-Aconitic acid	4.0×10^1	0	—	*1.5×10^5*	*$>1.5 \times 10^6$*
7	Isocitric acid	0	0	—	*2.8×10^5*	3.1×10^4
8	Lactic acid	*6.4×10^3*	3.4×10^3	0	*$>1.5 \times 10^6$*	4.4×10^4
9	Malic acid	8.0×10^1	*1.5×10^4*	5.8×10^3	3.6×10^3	5.0×10^4
10	Succinic acid	1.4×10^2	0	0	0	*1.4×10^5*
11	α-Ketoglutaric acid	2.1×10^2	0	5.1×10^2	0	*$>1.5 \times 10^6$*
12	Sodium fumarate	4.1×10^1	0	0	0	2.2×10^4
13	Mixture of: 2, 3, 4	1.0×10^2	0	—	2.5×10^1	5.1×10^4
14	Mixture of: 3, 4, 6	0	*2.4×10^4*	—	6.0×10^3	2.8×10^4
15	Mixture of: 7, 10, 11	0	0	—	0	*1.1×10^5*
16	Mixture of: 2 to 12	*2.1×10^6*	*2.5×10^5*	*1.1×10^6*	*$>1.5 \times 10^5$*	*$>1.5 \times 10^6$*
17	Nutrient broth	0	0	—	0	—

Contact time of bacteria with heat and various chemicals: (1) heat: 30 seconds at 71 C; (2) chlorine: (1 ppm) 6 minutes; (3) hydrogen peroxide: (0.3%) 25 minutes; (4) ethyl alcohol: (20%) 10 minutes; (5) "zephiran" chloride: ($1{:}1 \times 10^5$) 6 minutes.

jury by incubation with metabolites suggested studies regarding the injuries produced by various chemicals. From the practical point of view, the chlorine studies are most interesting and important since a major part of the water consumed by civilized populations is sterilized by chlorine treatment. Other chemicals of practical importance which were investigated were hydrogen peroxide, zephiran chloride, and ethyl alcohol. Data concerning these studies are summarized in table 3. Chlorine "sterilized" bacterial suspensions contain viable cells when incubated with sodium citrate, oxalacetic, lactic, and malic acids. The highest number of viable cells is obtained when incubation of "sterile" suspensions takes place in a mixture containing all listed metabolites, but no viable cells develop when incubation takes place in buffer or in nutrient broth. Many of the metabolites which were effective in producing recovery from heat injury are quite ineffective in eliminating the effect of chlorine action. It appears that chlorine action is more specific than heat action which is in general agreement with results observed in protein and enzyme chemistry. Hydrogen peroxide in 0.3 per cent concentration did not produce complete sterility after 20 minutes' contact time with bacteria. However, 24 hours' incubation with buffer sterilized the suspension suggesting that catalase did not decompose all of the hydrogen peroxide. Here also several metabolites are effective in producing cellular recovery; however, the mixture containing all metabolites is again most effective.

Zephiran chloride action on bacteria seems to be reversed very effectively by many metabolites. This suggests that the basic injury produced by zephiran chloride is relatively slight; however, suspensions when incubated in buffer or in nutrient broth remain sterile.

Ethyl alcohol concentration was kept relatively low (20 per cent) in order to avoid excessive

cellular injury. After ten minutes' contact time with alcohol, bacterial suspension was diluted in 1:10 ratio with buffer and one part plated immediately and the other incubated for 24 hours with metabolites. Under these conditions no viable cells were observed in any of the incubated samples. It was evident that residual alcohol (2 per cent) was capable of suppressing the cellular recovery. Earlier experiments had indicated that at such concentration ethyl alcohol did not have any significant effect on normal bacteria. Experiments were repeated, and after 20 minutes' contact time bacteria-alcohol mixture was diluted in 1:10 ratio with cold buffer (5 C) and dialyzed under sterile conditions. A small agitating type of electrophoresis dialyzer (Aminco) was used. Buffer was replaced three times after two hours of each dialyzing cycle. Bacteria were incubated subsequently in metabolites and plated. It is evident that buffer incubation alone is capable of restoring some number of viable cells, but addition of metabolites produces a broad spectrum of recovery and in some cases probably complete restoration of viability. This suggests that the action of alcohol at such concentrations produces only a very superficial cellular injury. In other experiments, where contact time was increased even to 25 minutes, a number of viable cells was produced when a sterile suspension was incubated with oxalacetic acid.

Effect of metabolites when present in agar M-9 plates. It was desirable to determine whether the addition of metabolites to the plating medium would induce cellular recovery. One part of sterile bacterial suspension (sterilized by various methods) was plated on supplemented, M-9 agar (table 3, mixture 16) and M-9 agar alone. The other part of the sample was incubated 24 hours in buffered metabolites (table 3, mixture 16) and plated out on M-9 agar. An increase of viable cell count or loss of sterility was observed only on occasions when treated bacterial suspensions were preincubated with metabolites, indicating that the presence of metabolites in agar plating medium is not inducive to cellular recovery. However, when bacteria after incubation with metabolites are plated on M-9 medium, which also contains metabolites, an increased number of colonies will develop. This phenomenon suggests that some bacteria during the incubation have adapted to the substrate as carbon source and will develop normal colonies when this substrate is present in the plating medium. Further studies are desired on this subject since restoration of cellular viability by adaptation to a new carbon source may be helpful for elucidating the mechanism of the reactivation process.

How is growth controlled or prevented when the "killed" bacteria are incubated with metabolite? In sterility experiments, bacterial samples after treatment with chemicals or heat were tested in several ways for viable cells: A large inoculum (1 ml, containing 1×10^6 cells) of treated suspension was used to seed nutrient broth and incubated for 24 hours. When no visible growth developed, the suspension in nutrient broth was tested for growth by plating 0.1 ml samples on M-9 agar. When no colonies appeared after 48 hours of incubation, the suspension was considered sterile. Further tests consisted of plating bacterial suspensions immediately after treatment with chemicals or heat and after incubation of samples in buffer for 24 hours at 37 C. Phosphate buffer (pH 7.0) is not toxic to partially inactivated cells as indicated by other numerous experiments (Heinmets *et al., in press*).

When all described tests did not produce viable cells, the suspension was considered to be sterile. It is obvious that when such sterile bacterial suspension is incubated with metabolites and viable cells appear, as indicated by plating, at least the first generation of cells has to recover before multiplication can take place. What about subsequent multiplication after the recovery? It is obvious that an increase of optical density has to take place when a large number of additional cells develop in bacterial suspension (for example: 50 per cent of original count). However, measurements do not indicate any increase of optical density. One of the possibilities might be that some disintegration of bacteria might take place when suspension is incubated with metabolites. This is contradicted by experimental evidence that optical density is constant when normal cells are incubated in metabolites or treated cells are incubated in buffer. Such data suggest that no significant multiplication will take place in bacterial suspensions while incubated with metabolites. However, there is direct evidence that bacterial growth and reactivation processes are different types of phenomena and reactivation can take place in conditions where growth is impossible. The fol-

lowing example will serve to illustrate the issue. When a bacterial suspension containing 7.8 log units of viable cells is irradiated with ultraviolet (a dose that reduces the viable log count to 3.7) and then is incubated with 0.01 M oxalacetate in the presence of 0.2 M sodium fluoride, a two log unit increase of viable cell count takes place. However, sodium fluoride at such a concentration is inhibitory to the growth.

In summary, when injured bacteria are incubated with metabolites, the increase of viable cell count is produced essentially by reactivation but not by multiplication.

DISCUSSION

Knox *et al.* (1948) working with *E. coli* demonstrated that chlorine in bactericidal concentrations reduced the activity of various sulfhydryl enzymes and other enzymes sensitive to oxidation. The action of quaternary ammonium compounds is not entirely clear, but complex formation between the components of the cell wall and surface active agent seems to be the primary step in the inactivation process (Lawrence, 1950). Heat, hydrogen peroxide, and alcohol are all capable of inactivating enzymes, but most of the work has been performed on isolated enzyme preparations. Relatively few studies have been performed with the enzyme systems of the entire cell which have been subjected to various modes of injury. Oliver (1952) found that the activity of the decarboxylating enzymes of *E. coli* ceased above 80 C but recommenced on a return to lower temperatures. He also observed that *E. coli* suspensions killed by heat and chemicals were still sterile after 7 days' incubation in culture medium but possessed almost normal decarboxylase activity which indicated that loss of viability does not represent the loss of all enzyme activities. It is obvious that some other enzymes may have been inactivated by heat action. For example, when *E. coli* are inactivated by ultraviolet irradiation, the ability to oxidize glucose is only slightly affected, but pyruvate oxidation is suppressed extensively (to be published). Our measurements indicate that over a million hypochloride molecules or ions are required to "kill" one organism (*E. coli*, strain B/r). Since chlorine reacts with cellular sites irreversibly, this suggests that a large number of enzymes are inactivated in the process of killing one bacterium. Calculations on the uptake of the other bactericidal agents used in these experiments indicate that a large number of molecules interact before bacterial inactivation results. An indication of the diversity and severity of cellular injury can be seen when data in table 3 are compared. Zephiran chloride and ethyl alcohol produce a less severe injury than chlorine.

The phenomenon that cells fail to grow or multiply in normal culture medium after subjection to chemical or heat injury but still exhibit some metabolic and synthetic activity suggests that the normal nutritional pathway(s) has become inoperative. However, under such conditions, the cell still could be functioning partially in processes involved in intermediary metabolism. It was speculated that, if a route of entry into respiratory cycle(s) could be found with a proper metabolite, a partial restoration of cyclic and interrelated processes could take place. Such a phenomenon could be visualized as a stepwise operation in a chain process where metabolite induces the enzyme formation and subsequent enzymatic conversion of metabolite would provide a new metabolite for other enzyme formation. In such a manner, a complete cycle could become operative. Such mechanism would be similar in principle to simultaneous enzyme adaptation as proposed by Stanier (1947).

If an extensive block would exist in one of the steps in the cycle, the inherent character of feedback mechanism of cyclic processes would permit an alternate route through a pathway in the reverse direction. Rate of such recovery seems to have an asymptotic characteristic. Obviously the possibility exists that a recovered cell may adapt itself to the original metabolite as carbon source. That such a phenomenon takes place is indicated by experimental evidence that the presence of the metabolite in plating medium produces higher rate of the reactivation. However, the fact that large numbers of cells are capable of growing nutrient agar plates containing only glucose as carbon source suggests that normal nutritional pathways again may have become operative during the process of reactivation. This suggests that pathways of glucose metabolism also could become operative during the establishment of cyclic processes.

What is the evidence for the existence of a respiratory cycle(s) in bacteria *E. coli*, and what influence does the abnormal functioning of the cycle(s) have on the division process of bacteria?

There is only a limited amount of data available in the literature on this subject. Umbreit's (1953) analysis suggests that citric acid cycle and possibly some other cycles are operative in *E. coli*. Nickerson and Sherman (1952) have shown that undividing filamentous bacteria (*Bacillus cereus*) produced by magnesium deficiency had much lower pyruvate oxidation rate than normally dividing cells. In view of such evidence, lowered or blocked reaction processes in citric acid cycle may lead to disability of the cell to divide; restoration of normal cyclic processes may help to restore cellular viability. New enzyme formation could be a part of such reactivation mechanism since there is enough evidence that cells rendered nonviable are capable of forming enzymes (Stephenson and Yudkin, 1936; Baron *et al.*, 1953). Since our experimental data reveal that metabolites belonging to the citric acid cycle were effective in restoring cellular viability, metabolic reactivation may be operative on the principles of cyclic processes. However, other modes of recovery may be possible, depending on the character of the injury pattern.

An alternative mode of action of metabolites in the reactivation of "killed" bacteria could be interpreted as providing a supply of more readily available energy. Johnson and Cohn (1952) studied the effect of various tricarboxylic acids on *E. coli*, strain 221, and found that in a one per cent glucose medium, malate, oxalacetate, fumarate, and citrate considerably increased the growth rate. Their findings indicate that the growth of *E. coli* in a glucose medium is limited by the inability of Kreb's cycle enzymatic system or a part of this system to provide some of the components of the tricarboxylic acid cycle in rates adequate for maximum growth. Tricarboxylic acids would serve as immediately utilizable compounds as energy sources.

If "killed" bacteria fail to grow because energy is not readily available, then the addition of suitable metabolites to the plating medium should provide substrates which are immediately utilizable as energy sources. Experiments indicate that no significant reactivation takes place under these conditions. Addition of metabolites to the liquid nutrient growth medium does produce viable cells, but here it is impossible to estimate the rate of recovery since recovery and growth processes are superimposed. Incubation of "killed" bacteria with metabolites for one or two hours prior to the plating does not produce viable cells or increase the viable cell count. Since this time is sufficiently long to establish the equilibrium between the cell and metabolite, it appears that longer contact time is essential for other reasons.

There are some practical aspects of the metabolic recovery phenomenon which deserve attention. At present the sterility of a medium is tested by methods involving culture plate or culture tube techniques. Table 3 indicates that treated bacterial suspensions which are shown to be sterile by direct plating or broth methods yield a large number of viable cells when incubated with metabolites. It is obvious that present concepts of sterility are entirely arbitrary. The metabolic reactivation phenomenon indicates that a particular medium tested for sterility by conventional methods may be rendered septic when it comes into contact under suitable conditions with known or unknown metabolites; i.e., vaccines and other sterile supplies. This indicates that sterility standards may have to be raised to levels where metabolic recovery also fails in order to secure complete sterility.

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SUMMARY

Experiments indicate that suspensions of *Escherichia coli*, strain B/r, which have been sterilized by the action of heat, chlorine, "zephiran" chloride, and ethyl alcohol contain viable cells when incubated with various metabolites of the tricarboxylic acid cycle. When such "sterile" suspensions are incubated in buffer or in nutrient broth, no viable cells can be demonstrated. In the present series, the following metabolites were most effective in producing reactivation: (1) Heat "killed": Sodium citrate, lactic acid, and oxalacetic acid; (2) Chlorine "killed": Sodium citrate, malic acid, and oxalacetic acid; (3) Hydrogen peroxide "killed": Sodium citrate, lactic acid, *cis*-aconitic acid; (4) Zephiran chloride "killed": Sodium citrate, lactic acid, *cis*-aconitic acid, and isocitric acid; (5) Ethyl alcohol "killed": *cis*-Aconitic acid, α-ketoglutaric acid, succinic acid, etc; (6) The combination of 11 metabolites produced the highest reactivation.

Reactivation processes of bacterial cells are discussed in terms of resynthesis of enzymes and reestablishment of cyclic processes.

It is indicated that conventional testing and culturing methods are not adequate to determine the levels of "complete" sterility.

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