DO BROTH CULTURE FILTRATES CONTAIN A BACTERIAL GROWTH-INHIBITING SUBSTANCE?

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In 1923, Besredka reported that staphylococcus broth culture filtrates contain a substance which inhibits the growth of the homologous organism, but which exerts little or no effect upon the development of heterologous bacteria. He claimed, further, that this substance, to which he gave the name "antivirus," originates as a result of the disintegration of the bacterial cell and is diffused into the surrounding medium. These conceptions are summarized in the following quotations:

Quand on filtre sur bougie une culture de Staphylocoques en bouillon, âgeé de 18 jours, on obtient un liquide qui, au premier abord, ne diffère pas sensiblement du bouillon ordinaire: . . . . ensemenceé avec des microbes variés, ce liquide donne des cultures pouvant presque rivaliser, quant à leur richesse, avec du bouillon normal. . . . . Seuls, les Staphylocoques—quelle qu'en soit l'origine—réensemencés dans ce liquide, n'y poussent pas: ils conservent leur vitalité, mais sont incapable d'y faire souche. . . . . La culture filtrée renferme donc une substance qui paralyse l'activité des Staphylocoques, aussi bien in vivo que in vitro. . . . . Enfin, comme l'indique son mode d'obtention, cette substance a le pouvoir de diffuser dans le milieu ambiant.

Besredka's belief that other bacteria can be used in the preparation of similar filtrates is indicated in a later publication (1930) as follows: "Les mêmes principes président à la préparation des autres antivirus—streptococcique, colibacillaire, typhique ou tuberculeux; seuls varient dans ces cas les milieux de culture et quelquefois la durée du séjour à l'étuve." The following excerpt states clearly that "antivirus" has its origin in the bacterial cell

(1925): "La dislocation des corps microbiens a pour résultat la mise en libertè d'antivirus; . . . . " Other methods of preparation are also cited (1930), whereby the liberation may be accomplished by autolysis, rapid disintegration, etc.

Weichardt (1927) found that undiluted filtrates were inhibitory and believed that the action was due to the presence of protein split products in certain concentrations and therefore non-specific. He showed, further, that the addition of the filtrates, in dilutions of 1:10 and 1:100, to a synthetic medium resulted in growth stimulation. Ninni and Molinari (1928) reported also that filtrates were inhibitory and reached the conclusion that the effect was due to products of protein decomposition. After adding 2 parts of ordinary broth to the filtrate they observed that there was normal development of the organisms. When broth and filtrate were diluted equally with water and when the filtrate was added to equal amounts of ordinary broth, the growth was less than that in normal broth. As a result, they believed that the inhibitory effect was not due, except in a small degree, to a diminution of nutritive materials. Their investigations, therefore, led them to conclude that the inhibitory effect of the filtrates was in direct relation to the quantity of products formed by protein decomposition, and in inverse relation to the nutritive needs of the organisms inoculated. In some previous work, the writer (1929) inoculated *Staphylococcus aureus* and *Escherichia communior* into homologous and heterologous filtrates and noted that the growth of the organisms under both conditions was much less than in normal broth. Broth digested by the use of non-bacterial enzymes also failed to support growth of *Esch. communior* and *Staph. aureus* to the same degree as the original bouillon. The interpretations of these results appeared to lend support to the conclusions of Ninni and Molinari. Schweinburg (1928), however, after adding normal broth to culture filtrates and observing abundant growth, reached the conclusion that the failure of organisms to grow in homologous filtrates is due to an exhaustion of nutritive materials. Chaillot (1930), on the other hand, reported inhibition of staphylococcus growth in a mixture of 5 cc. of broth and 5 cc. of the homologous filtrate. He stated

that, " . . . . l'action empêchant d'un antivirus n'est nullement la conséquence de l'appauvrissement du milieu en principes nutritives."

In the literature cited, there appear to be three conceptions as to the nature of the effect of broth culture filtrates on bacterial growth: (1) that an active inhibitory substance ("antivirus") is liberated from the bacterial cells (Besredka); (2) that inhibitory products are formed as a result of protein decomposition (Weichardt, Ninni and Molinari); and (3) that the effect is due to an exhaustion or diminution of food materials (Schweinburg).

Due to these conflicting reports, it was deemed advisable to obtain further experimental evidence upon the problem. The possibility that the entire phenomenon may be due to simple dilution or exhaustion of nutrient material led to the experiments described in the present paper. These investigations aimed, not only to control the question of dilution of nutrient material, but attempted also to separate the elements in the cells from those in the medium, and to determine the possible presence of inhibitory substances in each.

METHODS

In general, the technic used in testing the different preparations for inhibition of bacterial growth was as follows: Varying amounts of the filtrate to be tested were added to a series of seven test tubes containing nutrient broth; the final concentrations of filtrate in a series ranged from 5 to 95 per cent; a tube containing broth only, and one containing filtrate only were included in each series. Strict asepsis was always observed. The organism being tested for growth was then inoculated, and growth recorded the following day. Growth of the same organism in broth containing the above proportions of distilled water or saline was noted at the same time.

Estimations of the growth were made by comparing the turbidity of the various tubes with that in tubes of plain broth. In the earlier part of the work such readings were checked by use of the Gates apparatus (1920), and by direct counts. Results were compared with those obtained by a disinterested member of

the staff and were satisfactory. The readings given, therefore, are those made by turbidity comparisons, and are recorded as follows: 3+ = normal growth; 1+ sl. = slight growth; ± = growth doubtful; − = no visible growth.

The *broth* employed in all experiments was the usual laboratory meat extract bouillon (3 grams Liebig's beef extract, 10 grams Bacto-peptone, 5 grams sodium chloride, C.P., and 1000 cc. distilled water), adjusted to pH 7.2 to 7.4, and autoclaved at 15 pounds pressure for fifteen minutes. When necessary, the broth was passed through filter paper before sterilization.

The *reactions* of all broths and filtrates were adjusted to pH 7.2 to 7.4 before use.

Berkefeld N *filters* were used in all cases.

Controls for sterility of broth and filtrate, and for normal growth in plain broth were included in each series.

The *cultures* of *Escherichia coli* and *Staphylococcus albus* were obtained from the department stock collection. For inoculation, three to seven-hour broth cultures were transferred by pipettes. One drop of the young culture was added to each tube, care being taken to avoid contamination. No visible turbidity resulted from the inoculation of this amount of culture.

PREPARATION OF FILTRATES

1. Filtrates of altered broth

Artificially digested broth was obtained by adding 0.06 per cent dry pancreatin (Merck's U.S.P.), 0.14 per cent Na_2CO_3, and 0.09 per cent toluene to bouillon. The mixture was incubated at 37.5°C. for periods varying from seven to fourteen days. After incubation, the material was heated for thirty minutes at 100°C. to expel the toluene and to destroy enzymes, made up to the original volume with distilled water, and then passed through a Berkefeld filter. Undigested mixture was heated, filtered, and used as a control for normal growth.

Culture filtrates were prepared by inoculating flasks of broth with *Esch. coli*, and incubating, heating, and filtering simultaneously with the pancreatinized broth.

2. Filtrates of bacterial cell solutions

Solutions of bacterial cells were prepared in three ways: (*a*) by autolysis; (*b*) by mechanical shaking; and (*c*) by dissolution in sodium hydroxide. In all cases the bacterial cells were obtained by growing large quantities on plain agar for twenty-four hours at 37.5°C. The growth was washed off with 0.85 per cent salt solution, centrifuged and washed three times with saline, then resuspended in the solution desired. After solution of the cells had been brought about, the preparations were passed through Berkefeld filters. Biuret tests showed the presence of protein in the filtrates.

a. Autolysates were obtained by placing 1 per cent suspensions of *Esch. coli* and *Staph. albus* in sterile distilled water and saline. The preparations were allowed to incubate for seventy-three days at 37.5°C. They were shaken at frequent intervals to hasten disintegration. It has been shown that autolysis of bacteria may occur in two to ten days (Rettger (1904)). Streak plates were made from each suspension at frequent intervals. Plates made on the last day of incubation showed marked decreases in the numbers of viable organisms. Suspensions in saline showed greater decreases than those in distilled water.

b. One per cent suspensions of *Esch. coli* and *Staph. albus* were placed in a shaking machine and shaken for eighteen hours with glass beads. Stained preparations of the centrifuged debris showed very few intact cells, indicating that most of the bacteria had undergone disintegration.

c. A 1 per cent suspension of *Esch. coli* was made up in a buffered saline solution. The suspension was then treated by adding 5 per cent by volume of 15 per cent NaOH and the mixture heated over the direct flame until maximum clearing occurred. Plain broth, in buffered solution, and buffered saline solution were treated simultaneously with the coli suspension. It was found necessary to use buffered solutions as previous work showed that the reactions of such preparations were not stable. The buffer used was a mixture of Na_2HPO_4 and NaH_2PO_4 in a final concentration of 0.2 M. This solution was isotonic by calculation.

EXPERIMENTAL DATA

In a preliminary experiment, it was shown that pancreatin-digested broth and broth cultures of *Esch. coli* are so altered during two to four days' incubation that their filtrates fail to support the growth of either *Esch. coli* or *Staph. albus* to the same degree as normal bouillon. In the case of the coli culture filtrate this phenomenon appears between the first and second days with regard to the homologous organism, and between the second and fourth days for the heterologous organism. There is an almost complete absence of visible growth of both organisms in a filtrate obtained after seven days' incubation of the coli broth culture. Growth of *Esch. coli* and *Staph. albus* in filtrates from two- to four-day pancreatin-digested broth is definitely less than in normal broth or undigested pancreatinized broth. The lack of ability to support growth exhibited in a filtrate taken after seven days' digestion by pancreatin is not as marked as in the case of the coli culture filtrate, but is much more evident than at the end of the fourth day. Growth of both organisms in undigested pancreatin broth was equal to or even more abundant than that in plain broth.

The results of this experiment suggest that the failure of organisms to grow in broth culture filtrates is not due to a specific factor; they lead to the assumption that the effect is not necessarily dependent upon bacterial growth, but to an alteration of proteins in the menstruum as suggested by Ninni and Molinari (1928).

If no actually bacteriostatic substance is present, the addition of the filtrates in varying amounts to nutrient broth should result in no greater decrease in growth than the addition of ordinary distilled water. In other words, if the phenomenon is due to an exhaustion of food materials, the filtrates when added to broth should act merely as a diluent of the nutrient material. The following experiments were devised to determine this point.

EFFECT OF FILTRATES OF ALTERED BROTH

Filtrates of a fourteen-day coli broth culture and of fourteen-day pancreatin-digested broth were prepared as previously

described. These were added to nutrient broth in proportions varying from 5 to 95 per cent. Similar series were prepared by the addition of sterile distilled water. The various series of tubes

TABLE 1

Effect of addition of broth filtrates and distilled water on growth of Esch. coli and Staph. albus in nutrient broth

ORGANISM INOCULATED	SUBSTANCE ADDED TO BROTH	PERCENTAGE OF SUBSTANCES ADDED TO BROTH								
		Broth only	5 per cent	25 per cent	35 per cent	50 per cent	65 per cent	75 per cent	95 per cent	Filtrate only
Esch. coli, homologous	Coli broth culture filtrate	3+	3+	2–3+	2+	1–2+	1+ sl.	1+ sl.	1+ very sl.	1+ very sl.
	Pancreatin digested broth filtrate	3+	3+	3+	3+	3+	2–3+	2+	1+	1+ very sl.
	Distilled water	3+	3+	2–3+	2–3+	1–2+	1–2+	1+ sl.	± to −	−
Staph. albus	Coli broth culture filtrate	3+	3+	3+	3+	3+	3+	1+ sl.	1+ sl.	1+ sl.
	Pancreatin digested broth filtrate	3+	3+	3+	3+	3+	3+	2–3+	2+	1+
	Distilled water	3+	3+	3+	2–3+	2+	2+	1–2+	1+ sl.	−
Esch. coli, heterologous*	Coli broth culture filtrate	3+	3+	3+	3+	2–3+	2+	1–2+	1+ sl.	1+ very sl.
	Pancreatin digested broth filtrate	3+	3+	3+	2–3+	2+	2+	1+	1+	1+ very sl.
	Distilled water	3+	3+	2–3+	2–3+	2+	2+	1+	± to −	−

were inoculated with *Esch. coli* and *Staph. albus*. A third set of tubes was inoculated with a heterologous strain of *Esch. coli*. The results were observed the following day after incubation at 37.5°C., and are recorded in table 1.

It will be noted, in this table, that the decrease of growth caused by the addition of the broth culture filtrate was no greater than that effected by dilution of broth with ordinary distilled water. The same observation can be made with regard to the use of pancreatin-digested broth. It is also important to note that the homologous organism does not grow as well in broth diluted with the culture filtrate as do either of the heterologous organisms. This may be explained by assuming that organisms vary in their food requirements as regards amount and composition. Thus, it follows that a filtrate of a broth culture in which a given organism has been grown will contain less nourishment for the homologous species than for the heterologous type. The results shown in table 1 confirm this conception.

The results of this experiment fail to support the claim that broth culture filtrates contain a specific inhibitory agent originating as a result of disintegration of bacterial cells. They suggest, on the other hand, that the failure of organisms to grow in such preparations may be due to an exhaustion of necessary food substances.

EFFECT OF FILTRATES OF BACTERIAL CELL SOLUTIONS

It has been stated that the disintegration of bacterial cells results in the liberation, into the surrounding medium, of a substance capable of inhibiting the growth of the corresponding organisms. If this is true, filtrates of bacterial cell solutions added to nutrient broth should exhibit the phenomenon. Filtrates of the cell solutions previously described were accordingly added to broth in the manner employed in the preceding experiment. The results are shown in table 2.

The results given in this table show that no inhibitory agent was present in the cell solutions used. While there is a diminution in the growth of the organisms as the percentage of filtrate increases, the decrease is comparable to, or even less than, that exhibited when plain distilled water or saline is used in the same proportions. The experiment furnishes additional evidence against the assumption that the failure of organisms to grow in culture filtrates is due to products of cell disintegration.

TABLE 2

Effect of addition of filtrates of bacterial cell solutions on growth of homologous organisms in nutrient broth

ORGANISM INOCULATED	SUBSTANCE ADDED TO BROTH	PERCENTAGES OF SUBSTANCES ADDED TO BROTH								
		Broth only	5 per cent	25 per cent	35 per cent	50 per cent	65 per cent	75 per cent	95 per cent	Filtrate only
Staph. albus	Staph. autolysate in distilled water	3+	3+	3+	3+	2-3+	1-2+	1-2+	±	−
	Staph. autolysate in saline	3+	3+	3+	3+	2-3+	2+	2+	±	−
	Plain distilled water	3+	3+	3+	2-3+	2-3+	2+	1+	±	−
Esch. coli	Coli autolysate in distilled water	3+	3+	3+	3+	3+	2-3+	1+	± to +	−
	Coli autolysate in saline	3+	3+	3+	3+	2-3+	2+	1-2+	± to +	−
	Plain distilled water	3+	3+	2-3+	2+	2+	2+	1+	−	−
Staph. albus	Shaken staph. cell solution	3+	3+	3+	3+	2-3+	2-3+	1+	±	−
	Saline	3+	3+	3+	2-3+	2-3+	2+	1+	1+ very sl.	−
Esch. coli	Shaken coli cell solution	3+	3+	3+	2-3+	2+	1-2+	1+	1+ very sl.	± to −
	Saline	3+	3+	3+	2-3+	2+	1-2+	1+	1+ very sl.	−
Esch. coli	NaOH dissolved coli cell solution	3+	3+	2-3+	2-3+	2+	2+	1+	± to −	± to −
	NaOH treated broth	3+	3+	2-3+	2-3+	2-3+	2+	1-2+	1+ sl.	± to −
	NaOH treated saline	3+	3+	2-3+	2-3+	2+	1-2+	1+	± to −	± to −

The experiments described in this paper were repeated a sufficient number of times to rule out experimental error. Results were always comparable to those cited.

DISCUSSION

The fact that broth culture filtrates fail to support normal growth of bacteria is well known. Besredka (1923, 1930) has attributed this effect to the hypothetical presence of an inhibitory substance. He believes that this property is specific for the organism concerned, and arises as a result of disintegration of the bacterial cell. Ninni and Molinari (1928), on the other hand, have reported conflicting observations. Their evidence led them to conclude that the failure of organisms to grow in broth culture filtrates was due to an accumulation of products of proteolytic decomposition of the medium. They believe, further, that the effect is not specific, and varies according to the digestive activity of the organism used. These authors did not deny the existence of inhibitory properties in culture filtrates.

Evidence in support of these conceptions depends, in part, upon the following considerations: (1) It must be shown that broth culture filtrates will support the growth of certain heterologous organisms but not of homologous bacteria; (2) the presence of an actively bacteriostatic substance in such filtrates must be demonstrated; (3) preparations of disintegrated bacterial cells should inhibit the growth of the homologous organisms; (4) diminution of bacterial growth in nutrient broth diluted by the filtrates should be appreciably greater than that following the addition of water or saline in the same proportions.

The results of experiments described in this paper show that: (1) Broth, digested by pancreatin under the conditions described, did not support the normal growth of *Esch. coli* or *Staph. albus;* (2) broth culture filtrates of the organisms used failed to support normal growth of homologous and certain heterologous bacteria; (3) filtrates of solutions of disintegrated bacterial cells caused a diminution in growth of homologous organisms as the concentrations of filtrates increased; (4) in no case did the decrease in growth of organisms in broth diluted with culture filtrates or cell

solution filtrates exceed that exhibited when broth was diluted in the same proportions with distilled water or saline; in most instances the growth was less under the latter conditions. These findings disagree with Besredka's claims regarding the presence of an inhibitory agent in broth culture filtrates,—they lead to a denial of the existence of such a factor. There is a disagreement also with the report of Ninni and Molinari in so far as the presence of an inhibitory substance is concerned, but agreement with those authors in explaining the *apparent* inhibitory effect upon the basis of an alteration of proteins in the medium. The work of Butterfield (1929) on the relation between food concentration in liquid media and bacterial growth is of interest in connection with the observations reported in this paper. He found that an increase in food concentration, within certain limits, resulted in an increase in the numbers of organisms which developed.

These observations have a bearing on certain conceptions of local immunization. Besredka believes that broth culture filtrates possess local immunizing properties specific for the organism grown in the bouillon. Experimental results published by Gay and Morrison (1923), Freedlander and Toomey (1928), and others, indicate that the power of such filtrates to stimulate the production of local immunity is not specific. The effect is rather one of local response to irritants of a non-specific nature. The local immunizing effect of certain filtrates described in this paper remains to be determined.

CONCLUSIONS

The results of experiments described in this paper do not support the observations of Besredka that broth culture filtrates contain a bacterial growth-inhibiting substance liberated from the microbial cell.

When filtrates, prepared from broth cultures, pancreatin-digested broth, or bacterial cell solutions, are added in varying proportions to nutrient broth, the resulting diminution in growth of inoculated organisms is comparable to, or even less than, that observed when distilled water (or saline) is used as the diluent.

It appears to the writer, therefore, that the effect of culture

filtrates on the growth of bacteria is due to an alteration of necessary nutrient materials rather than to the presence of a truly inhibitory agent.

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