CHEMICAL INVESTIGATIONS ON THE ACTIVE PRINCIPLES OF THE PHENOMENON OF LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES

III. Application of Dialysis to the Production of the Active Principles in Fluid Media*

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It is known that the yield of staphylococcus toxin in plain broth and other fluid media is poor (1). The use of solid or semisolid agar media appears necessary for producing active preparations. Recently, however, McClean (1) was able to obtain potent toxins by the use of fluid media diffused through cellophane bags. The surface of the cellophane membrane appeared to play a rôle similar to that of the agar.

It was our consistent observation (2) that "agar washings" contain the most potent substances necessary for eliciting the phenomenon of local skin reactivity to bacterial filtrates. It was of interest, therefore, to investigate the production of these substances when various bacteria were grown in the diffused medium described by McClean. The possibility of applying this method successfully was suggested by the previous observation that the active principles of the phenomenon were retained by cellophane membranes (3).

The present paper embodies experiments on (*a*) the production of the active principles from various microorganisms grown in the diffused broth medium; and (*b*) the purification attained when culture filtrates so obtained were redialyzed.

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EXPERIMENTAL

1. Production of Active Principles of Bacillus coli, Meningococcus 44B., and Bacillus typhosus Grown in Broth Media Diffused through Cellophane.—The technique described by McClean (1), who grew staphylococcus in a diffused fluid medium, was first used, but contaminations of the broth outside the cellophane membrane were

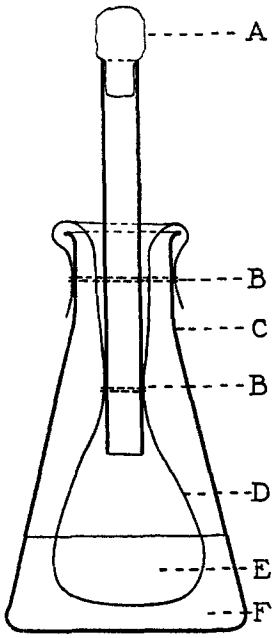

CHART 1. Apparatus for cultivation of bacteria in diffused broth medium. (A) Cotton plug. (B) Rubber bands. (C) Flask. (D) Cellophane bag. (E) Saline. (F) Broth.

frequently encountered. A somewhat modified procedure was, therefore, developed as described below (see Chart 1).

A sheet of plain transparent "Cellophane,"[1] No. 600, (50 x 50 cm.) was cut circularly and thoroughly wetted in a large evaporating dish. The membrane was drawn evenly around a glass tubing (150 x 18 mm.) to form a bag, and

[1] We wish to express our thanks to E. I. Du Pont De Nemours and Company, Cellophane Division, New York, for their cooperation in supplying us with various grades of cellophane.

wrapped tightly with several rubber bands near the bottom of the tube. The bag was then immersed in a flask of broth and the membrane wrapped tightly with rubber bands on the outside of the flask. The tube was plugged with non-absorbent cotton and the entire apparatus autoclaved for 20 minutes under 15 pounds of steam pressure. When the flask had cooled, sterile saline was pipetted into the bag. After standing at room temperature for 24 hours, the solution inside the bag, referred to as the *diffusate medium*, was inoculated and the apparatus incubated at 37°C. for various periods of time. The contents of the bag were removed with a pipette and filtered through Berkefeld V candles.

A plain veal infusion broth, containing 1 per cent peptone (Difco) was the basic medium for all the experiments. The proportion of broth to saline was 2:1, as suggested by McClean (1). Small and large assemblies were set up. For the former, a 250 cc. flask containing 100 cc. of plain broth was used, 50 cc. of saline being introduced inside the bag. For the latter, a 100 x 100 cm. cellophane sheet was employed for preparation of bags. The bags were immersed in 2 liter flasks containing 1 liter of broth, and 500 cc. of saline were introduced into them. Wherever indicated (Table I), the broth was enriched with glucose (to 0.5 per cent), glycerol (to 0.5 per cent), and Na_2HPO_4 (to M/100). Suction flasks were employed whenever it was desired to add sterile ingredients which might be affected by autoclaving, like whole blood, serum, cocoanut milk, etc. The side arms, plugged with cotton, provided a convenient means for addition of these materials, after the apparatus was autoclaved.

B. coli, strain Lewis, and *B. typhosus*, strain T_L, were grown for 24 hours on plain agar, and meningococcus, group III, 44B., on North agar slants. Suspensions were prepared by washing each slant with 5 cc. of saline. The size of the inoculum varied with the different organisms: for *B. coli*, 0.1 cc. of a 1:100 saline dilution; for *B. typhosus* and meningococcus, 0.1 cc. and 1.0 cc. of the undiluted saline washings, respectively. Any variations from these inocula are noted in Table I.

About fifty culture filtrates have thus far been prepared in the diffusate media. Even after a week of incubation, the organisms did not grow through the cellophane membranes, as the outside broth remained sterile. In only several cases was the outside broth accidentally contaminated, with the inoculum or other bacteria, especially when ingredients were added after autoclaving. Whenever a contamination occurred, either in the diffusate or broth medium, the experiment was repeated.

The growth occurring under these conditions was extremely abundant. With *B. coli* and *B. typhosus*, a massive precipitate of organisms accumulated at the bottom and on the sides of the membranes. Even with meningococcus, the growth was considerable, appearing

TABLE I

Bacillus coli, Meningococcus, and Bacillus typhosus Active Principles Produced in Diffusate Media

Inoculum	Plain broth outside cellophane bag		Samples removed from inside cellophane bag			Preparation No.	Reaction in rabbits	
	Volume	Supplemented with	Time	Volume	pH		Dilution tested	Results*
	cc.		*days*	*cc.*				
B. coli (Lewis)	1000	Glucose, glycerol, phosphate	1	150	5.8	T.2444	1:25	±/2
			2	10	8.0	T.2465	1:25	0/3
							1:25	3/0
			6	50	7.0	T.2451	1:100	1/2
							1:200	0/3
			11	150	7.0	T.2467	1:25	2/1
	1000	Same as above, but medium removed from bag before inoculation	2	185	7.0	T.2487	1:25	0/3
			6	185	6.0	T.2499	1:25	1/2
	100	Glucose, glycerol, phosphate	3	8	4.8	T.2416	1:25	2/1
			7	20	5.0	T.2430	1:25	3/0
							1:25	3/0
	1000	" "	6	300	7.0	T.2496	1:75	1/2
							1:100	0/3
	1000	—	6	380	7.5	T.2573	1:25	1/2
	1000	Glucose, glycerol	6	400	5.2	T.2575	1:25	2+/2
Meningococcus (44B.)	100	5 cc. rabbit blood; 5 cc. cocoanut milk	2	8	7.5	T.2534	1:100	0/3
			6	16	7.5	T.2538	1:100	3/0
	100	Glucose, glycerol, phosphate; 5 cc. rabbit blood†	2	10	7.0	T.2431	1:300	1/2
			6	18	7.3	T.2436	1:750	3/0
							1:2000	2‡prim/1
	100	" "	2	8	7.5	T.2535	1:100	1/2
			6	22	7.0	Ch.402§	1:100	3/0
							1:1000	2‡prim/1

* The numerator indicates the number of positive rabbits. The denominator indicates the number of negative rabbits. The sum total of both indicates the total number of rabbits used in each group.

† Inoculum changed to 3 cc. of a 24 hour glucose (0.7 per cent) broth culture containing 1 per cent rabbit blood.

‡ Primary reactions from intradermal injections.

§ Culture filtrate was dialyzed before testing.

TABLE I—*Concluded*

Inoculum	Plain broth outside cellophane bag		Samples removed from inside cellophane bag			Preparation No.	Reaction in rabbits	
	Volume	Supplemented with	Time	Volume	pH		Dilution tested	Results*
	cc.		*days*	*cc.*				
Meningococcus (44B.)	1000	Glucose, glycerol, phosphate	6	300	7.5	T.2586	1:100	0/3
	1000	Glucose, glycerol; 10 cc. rabbit blood	6	380	7.5	T.2587	1:100 1:500	2/1 0/3
B. typhosus (T_L)	100	Glucose, phosphate; 5 cc. rabbit serum	3	10	5.3	T.2433	1:50	0/3
			5	16	5.5	T.2435	1:50	2/1
	100	Phosphate; 5 cc. cocoanut milk	2	10	7.0	T.2489	1:50	0/3
			5	12	7.5	T.2495	1:50	0/3
	100	10 cc. 10 per cent gelatin	2	18	7.0	T.2514	1:50	1/2
			6	18	7.5	T.2521	1:50	2/1
	1000	—	6	280	8.0	T.2576	1:50	0/3
	1000	10 cc. rabbit blood	6	300	8.0	T.2577	1:50	1/2

at about the 4th day. The viability of the growth inside the bags was controlled, in many instances, by daily subcultures and spreads. The cultures apparently remained viable during the incubation periods described. Although meningococcus subcultures grew well throughout the entire period of incubation, they occasionally appeared autolyzed, on spreads, at about the 6th day.

Results of experiments on the phenomenon-producing potency of various preparations are summarized in Table I. Groups of three rabbits were injected intradermally with 0.25 cc. of the filtrate under test, and 24 hours later, intravenously with 1 cc. per kilo of body weight, of the various dilutions indicated. The production of substances which are active in eliciting the phenomenon under discussion has been consistent. As may be seen (Table I), no significant differences were noticed in the potency of the *B. coli* and *B. typhosus* diffu-

sate filtrates prepared in either the small or large assemblies. With meningococcus, however, the highest titers were obtained with the smaller set up (100 cc. plain broth). In all cases, a 6 day incubation period gave the best results, when, as noted above, cultures were still viable and little evidence of autolysis was seen on spreads. Inactive preparations frequently resulted after shorter periods of incubation. Although active *B. coli* preparations were obtained with the basic medium alone (T.2573), addition of glucose, glycerol, and phosphate seemed to enhance their potency (T. 2451). With *B. typhosus*, rabbit serum (T.2435), rabbit blood (T.2577), or gelatin (T.2521) were apparently required, whereas with meningococcus, the presence of blood (compare T.2586 where blood was absent) was a necessary factor.

The presence of antigenic substances in the culture filtrates was established by precipitation and neutralization titrations with specific antisera. For example, *B. coli* filtrate T.2496 (Table I) showed a precipitation titer of 1:32 with an anti-*coli* horse serum (H.492 prepared and concentrated by Eli Lilly and Company). 0.50 cc. of the serum completely neutralized 250 reacting units of the culture filtrate. It is of interest to note that the same precipitation and neutralization titer was obtained on this serum with a *B. coli* culture filtrate grown in synthetic medium broth (T.2158, formerly described (3)).

2. Dialysis of Culture Filtrates Grown in Diffusate Media.—The technique for dialyzing culture filtrates under strictly sterile conditions has already been described (3). In our former experiments, distilled water was used outside the cellophane bags. In the present investigation, however, 0.85 per cent sodium chloride has been employed, since the volume changes occurring in the bags were negligible under these conditions. The solutions were rendered isotonic during the dialysis and thus ready for injection. Furthermore, the rubber stoppers and glass supports (3)[2] were replaced by rubber bands, which proved more convenient and equally efficient with respect to sterility. The total solids and nitrogen analyses were conducted as formerly described. Ash was determined by heating to a dull red in a muffle oven; constant weights were obtained in a few hours. Correction of the total solids to an ash-free basis was necessary, since saline was used in conducting the dialyses. The ash content of the dialyzed solutions was always found equal to the sodium chloride concentration used for dialysis.

[2] Shwartzman, Morell, and Sobotka (3), page 331.

When the culture filtrates from the diffusate media were dialyzed, clear, slightly colored solutions were obtained which not only possessed all of the activity originally present, but also contained extremely small amounts of dissolved substances. Filtrates from *B. coli*, grown in various diffusate media, have been analyzed, before and after dialysis, for their total solids, ash, and nitrogen contents. The extent of purification was thus determined. There was also obtained a better understanding of the processes involved in the production of the active principles under the conditions described.

As already noted, the apparatus was allowed to stand at room temperature for 24 hours previous to inoculation. During this time various broth ingredients diffused into the saline in sufficient concentration to initiate bacterial growth. Analyses of the inner contents of the bags were conducted prior to inoculation, as well as after various periods of time following inoculation and filtration. The broth outside the bags was similarly analyzed. In one case, the diffused medium was removed from the cellophane bag, after the 24 hour period, and inoculated in an Erlenmeyer flask, thus preventing further diffusion of broth ingredients. The analytical values obtained from plain broth culture filtrates were corrected for the 1:1.5 dilution which the presence of saline produced in the diffusate culture filtrates. The results of these analyses have been summarized in Table II. Since routine preparations of broth may vary considerably, the same batch was used for the experiments compared under section A of Table II; a similar batch of broth was used for all of the experiments described under section B.

It can be seen (section A of Table II) that before inoculation, the diffusate medium (Ch.337/371) contained, after dialysis, only 0.8 and 0.01 mg. per cc. total solids and nitrogen, respectively. When inoculated and incubated for 6 days (T.2451/Ch.336) these values increased to 1.3 and 0.17, respectively, thus indicating that an appreciable amount of non-diffusible materials, particularly nitrogenous, had formed during growth. The latter figures were in agreement with those obtained in the diffusate medium removed from contact with the outside broth (T.2499/373), namely, 1.1 and 0.10, respectively; under these conditions there was a much lighter bacterial growth. A comparison of the outside broth before inoculation (Ch.338/372), to

TABLE II

Total Solids and Nitrogen Analysis of Bacillus coli Culture Filtrates before and after Dialysis

Section	Filtrate No.	Description	Incubation	Total solids* per cc.		Total nitrogen per cc.	
				Before dialysis	After dialysis	Before dialysis	After dialysis
			days	*mg.*	*mg.*	*mg.*	*mg.*
A†	Ch. 337/371	Diffusate medium removed before inoculation	—	15.9	0.8	1.89	0.01
Comparison of diffusate medium to broth outside cellophane bag, before and after inoculation	T.2451/Ch.336	Diffusate medium retained in bag and inoculated	6	12.1	1.3	1.98	0.17
	T.2499/Ch.373	Diffusate medium removed from bag and inoculated in Erlenmeyer flask	6	12.3	1.1	1.89	0.10
	Ch. 338/372	Broth from outside bag of Ch.337/371	—	26.1	8.0	3.47	1.20
	Ch. 336/367	Broth from outside bag of T2451/Ch366	6	14.3	4.0	2.23	0.59
B†	T.2486/Ch.380	Diffusate medium	2	14.6	1.4	1.98	0.18
Comparison of preparations in diffusate medium, plain broth, and uninoculated diffusate controls	T.2496/Ch.381	" "	6	14.1	1.9	2.25	0.29
	T.2515/Ch.384	Plain broth	2	15.5	6.3	2.48	0.91
	T.2532/Ch.386	" "	6	14.8	4.5	2.51	0.71
	Ch. 360/374	Uninoculated diffusate (control)	2	14.8	1.6	1.90	0.18
	Ch. 361/375	" "	6	16.8	1.9	2.33	0.21

* Ash-free basis.

† Different batches of broth were used for the preparations described in sections A and B, respectively.

that after *B. coli* grew inside the bag for 6 days (Ch.336/367), showed that a considerable quantity of diffusible materials was continually withdrawn during the incubation period. An approximately twofold

difference was evident, both before and after dialysis; this decrease probably represented the amount of material utilized during the abundant bacterial growth.

Advantages in using the diffusate as a fluid medium, rather than plain broth, can be seen from the comparison summarized in section B of Table II. In the diffusate medium cultures (T.2486/Ch.380 and T.2496/Ch.381), the total solids were reduced by dialysis from about 14 to 1.5 mg. per cc., and the total nitrogen was decreased from about 2 to 0.2 mg. per cc. In the plain broth cultures (T.2515/Ch.384 and T.2532/Ch.386), the analytical results were approximately the same as above, before dialysis; after dialysis, however, the total solids and nitrogen were reduced to only 5.4 and 0.8 mg. per cc. (average for the 2 and 6 day periods), respectively, which is about four times greater than the figures obtained above from the diffusate medium cultures. It is of interest to note that in the uninoculated diffusate controls (Ch.360/324 and Ch.361/375), the analytical results obtained after dialysis were practically identical to those obtained above for the inoculated media. During the 6 day incubation period, therefore, approximately the same amount of material was rendered non-diffusible in the presence or absence of bacterial growth. This amount, nevertheless, is considerably smaller than that obtained in the plain broth cultures.

DISCUSSION

As described in a previous communication (3), an appreciable purification of various bacterial filtrates, which were active in eliciting the phenomenon of local skin reactivity, was attained by means of dialysis. The membrane employed was cellophane. In addition to the active principles, however, the dialysate[3] also contained other soluble substances, from the broth and agar of the medium.

The observations of McClean (1), on the production of staphylococcus toxin in a broth medium diffused through cellophane, suggested the possibility of preparing active principles of the phenomenon of local skin reactivity free from non-specific ingredients. It appeared

[3] "Dialysate" is the portion of the original material which remains after dialysis; the crystalloid portion which passes through the membrane is termed the "diffusate" (1).

likely that if growth occurred in the diffusate, then redialysis of the filtrates of these cultures would yield preparations containing only polymeric non-diffusible substances elaborated by the growing cells, including particularly the active principles. The experiments described above have shown that this has essentially been accomplished. In this connection it is of interest to note that Pappenheimer, Mueller, and Cohen (4) have recently cultivated *B. diphtheriae* in a chemically defined synthetic medium and obtained potent toxin preparations.

The growth which occurred in the diffusate was extremely abundant and the production of the active principles very consistent. The presence of antigenic substances was established by repeated precipitation and neutralization titrations with specific antisera. Thus far, the titers of the filtrates tested were maintained for about 6 months. Tests for reacting potency were also made with various broth samples obtained from the outside of the cellophane bags, within which active preparations of *B. coli*, meningococcus, and *B. typhosus* had been produced. In all cases, the outside broth samples were completely inactive. It appears, therefore, that the active principles remained non-diffusible during their production by the growing microorganisms.

The advantages derived from the method described (*i.e.*, cultivation in diffusate media, and redialysis of the filtered cultures) are considerable. From the practical standpoint, the procedure of pooling the saline washings from hundreds of agar plates is laborious compared to the relative ease of producing equally large volumes in the diffused fluid media. It must be emphasized, however, that at the present stage of development, replacement of "agar washings" is not suggested, since the latter still yield consistently higher reacting titers. From the standpoint of further chemical investigations, however, the method is invaluable, since considerably purified starting materials are now readily available in large quantity.

SUMMARY

B. coli, meningococcus, and *B. typhosus* have been cultivated in a diffused broth medium, prepared by immersing cellophane bags containing saline into nutrient broth. An abundant growth occurred inside the bags, while the outside broth remained sterile. Under these conditions, the production of the active principles of the phe-

nomenon of local skin reactivity to bacterial filtrates has been consistent.

When the culture filtrates so obtained were redialyzed against saline, active preparations were obtained which were practically free from non-specific ingredients.

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