A STUDY OF FLUID THIOGLYCOLLATE MEDIUM FOR THE STERILITY TEST

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The use of sodium thioglycollate in a clear medium for the cultivation of anaerobic bacteria was introduced by Brewer in 1940. The following year the National Institute of Health adopted a liquid medium designated "fluid thioglycollate medium" for use in the testing of biological products for sterility. Two formulae were recommended. They differed only in that one (Brewer, 1941) specified beef infusion and 1.0 per cent of peptone, and the other (Linden, 1941) specified 2.0 per cent of peptone and 0.2 per cent of yeast extract but no meat infusion.

In replacing the infusion broth, previously recommended by the National Institute of Health, with the fluid thioglycollate medium two distinct advantages were apparent: (1) neutralization of the bacteriostatic action of the mercurial preservatives and (2) provision of both aerobic and anaerobic conditions in one test tube. The advantage of the neutralization of the bacteriostatic effect of the mercurial compounds has been emphasized by Nungester *et al.* (1943). The varying oxidation-reduction conditions in a single culture tube renders the medium valuable for diagnostic use (Foley and Schaub, 1944).

Subsequent use of the thioglycollate medium for the sterility test demonstrated that it was superior to the infusion broth. In parallel tests with the two media, Dr. H. C. Batson (personal communication) detected the presence of many more contaminants with the use of the new medium. A few contaminants, however, developed only in the broth. He found that the latter were inhibited by the methylene blue used as an Eh indicator in the thioglycollate medium. Dr. Geoffrey Edsall (personal communication) reported similar observations; and we confirmed their findings. This inhibitory action could be reduced but not entirely eliminated by enrichment of the medium.

The purpose of the present investigation was to develop a formula for the sterility test medium which would support the growth of the greatest possible variety of bacteria when cultured at the optimum temperature. In accomplishing this it was hoped that it would be possible to use only ingredients lending themselves readily to exact definition and reproducibility, and obtainable without restriction on the open market. Particular emphasis was placed upon the choice of a peptone and an oxidation-reduction indicator, as well as the determination of the optimum amount of yeast extract, sodium chloride, and *l*-cystine, and also the most favorable pH.

During the investigation many people were consulted; from them valuable suggestions and assistance were received. To all are expressed thanks and appre-

ciation. Among those deserving special mention are Dr. John H. Brewer for his constant interest, particularly in reference to the peptone and the chemical tests for the purity of sodium thioglycollate and thioglycollic acid; Dr. J. Howard Brown for tests to determine the digestion products and the growth-promoting action of the pancreatic digest of casein (peptone); Mr. H. G. Dunham, of Difco Laboratories, for preparation of some experimental media and for the suggestion to use resazurin; Mr. Theo. J. Carski, of Baltimore Biological Laboratory, for preparation of certain experimental media; Dr. Harriette D. Vera for the suggestion to use *l*-cystine; Dr. J. G. Brereton, of Sheffield Farms Company, for interest in the production of pancreatic digest of casein; Mr. B. A. Linden for suggestions and co-operation in some duplicate tests; and Dr. Milton V. Veldee for assistance in planning the work.

METHODS

Media. The formulae of the experimental media or the variations from the final revised formula are given with the protocols of the experiments. The revised formula as finally adopted is as follows: *l*-cystine, 0.75 g; sodium chloride, 2.5 g; glucose, 5.0 g; agar, 0.75 g; water-soluble extract of yeast, 5.0 g; pancreatic digest of casein (peptone), 15.0 g; sodium thioglycollate, 0.5 g, or thioglycollic acid, 0.3 ml; resazurin, 1.0 ml of a 0.1 per cent solution freshly prepared; and distilled water, 1 L.

When *l*-cystine was first included in the experimental media, some difficulty was experienced in dissolving it. This was overcome by mixing the dry ingredients, except the sodium thioglycollate, in a mortar in the order given above. Each was thoroughly mixed as it was added. Then a portion of the water, previously heated, was added with stirring, and the resulting paste transferred to a suitable flask. The remainder of the water was added. The solution was completed in a steam bath. Then the sodium thioglycollate or thioglycollic acid was added, the pH was adjusted with sodium hydroxide, and finally the resazurin was added. The medium was dispensed in 15-ml amounts in 20 × 150-mm test tubes and sterilized in the autoclave for 18 minutes at 120 to 123 C.

The pH of the finished media used in the greater part of the study was 7.2 ± 0.1. An analysis of many experiments revealed that better growth had been obtained in those media having a pH of 7.1 than in those having a pH of 7.2 or 7.3. This was confirmed by further experimentation.

Cultures. The cultures used in the study either had been isolated from contaminated biological products or were considered as possible contaminants, and were very largely selected for their exacting growth requirements. It is imperative that an all-purpose sterility test medium should be capable of initiating the development of *Clostridium tetani* and other pathogenic anaerobes. Cultures of certain other species of *Clostridium* were also used because of their peculiar growth requirements.

For a particular experiment, the cultures were selected which were the most exacting toward the ingredient under study. If any changes were indicated to

meet the growth requirements of a fastidious organism, it was always ascertained that the most commonly occurring contaminants, gram-positive cocci, diphtheroids, sporeforming aerobes, and bacteria of the coli-aerogenes group and of *Pseudomonas*, would in no wise be hindered in their development by these changes.

A list of the cultures is given below. Some of the cultures have not been fully identified, but they have been placed with that genus or group to which they appear to be most closely related.

Aerobacter	
cloacae	PC3B
Bacillus	
cereus	PC3A
*subtilis**	Peoria
*Clostridium**	
acetobutylicum	824
	6085
butylicum	37
	Yale
chauvoei	Mont.
	2585
novyi	140
	N.I.H.
perfringens	SR12
sporogenes	N.I.H.
tetani	Tullock
Corynebacterium	
*pseudodipheriticum**	(Mass.)
species (?)	PC20
	PC32
	PC33B
	PC96
	Mich. 61
Gram-positive rods, genus (?)	
orange-pink pigment	Mich. 6
red pigment	Mich. 14
curved forms	PC66
Micrococcus	
epidermidis	PC8
roseus (?)	Mich. 57
rosaseus (?)	Mich. 58
species (?)	
no pigment	PC99
pale yellow pigment	Mich. 14
pink pigment	Mich. 38
orange-pink pigment	Mich. 54
lemon-yellow pigment	Mich. 60
Micromonospora	
	Mich. 48
	Mich. 53
*Diplococcus pneumoniae**	
type 3	
type 17	
type 37	
Pseudomonas	
ovalis	PC25
species (?)	PC84
species (?)	I60 (Mass.)
Staphylococcus	
aerogenes	PC1
*aureus**	Cumming
epidermidis	PC63
Streptococcus	
liquefaciens	PC16
*pyogenes**	NY5
species (?)	PC22
species (?)	PC33A

* Not isolated from contaminated biological products.

Preparatory to inoculating the media the aerobic cultures usually were grown on agar slants; a few were grown in broth. The anaerobic cultures either were grown in a cooked meat medium or a fluid thioglycollate medium. Overnight incubated cultures were used when possible; some required longer incubation. Suspensions of the agar slant cultures as well as the other cultures were diluted tenfold serially in 0.4 or 0.85 per cent sodium chloride solution until just less than one bacterium was present per ml. The highest 5 or 6 dilutions were used for inoculating each medium under test. The inoculum was 1.0 ml per tube. After inoculation the tubes were twirled for thorough mixing and then incubated at 34 to 36 C or at room temperature for 7 days. Two *Pseudomonas* cultures grew very poorly at 34 C and not at all at 37 C. Examination of the tubes for visible growth was made at the end of 1, 2, 4, and 7 days. The degree of turbidity was recorded as 1+, 2+, 3+, and 4+.

EXPERIMENTAL

Eh Indicators

The bacteriostatic or inhibitory action of methylene blue against 8 cultures is shown in table 1. In columns 2 to 5 are recorded the number of tubes, inoculated with 6 dilutions of the respective bacterial suspensions, which showed growth of the bacteria in the medium without and with indicators. There was growth in a total of 28 tubes of medium containing no indicator (column 2) in contrast to a total of 8 tubes of the same medium to which methylene blue had been added (column 3). The same degree of inhibition was obtained in the presence of the zinc chloride salt of methylene blue (see columns 4 and 5). In contrast, there was no inhibition in the presence of resazurin, as shown in column 6.

The bacteria of a large number of species were cultured in the presence of resazurin. Growth was the same as in the medium containing no resazurin, excepting with a few with which the results were equivocal. The latter were cultured in concentrations of resazurin varying from 1:1,000,000 to 1:100,000. A summary of these results is given in table 2. The aerobic cultures were not inhibited in concentrations of 1:250,000 or less, but in 1:100,000 there was retardation in development. On the other hand, the anaerobic cultures were not retarded in the highest concentration, 1:100,000; in fact, some were definitely stimulated by the greater amounts of resazurin. These results suggest that there would be no contraindication to the use of resazurin in a concentration of 1:250,000 or less. A concentration of 1:1,000,000 seemed to be sufficient for indicating the oxidation-reduction conditions of the medium. This amount was less than one-fourth of that which inhibited the development of the most sensitive bacteria that we tested.

It should be mentioned that resazurin in a neutral solution is blue. In the presence of a reducing agent it is reduced to resorufin, which gives a pink color, and resorufin is reduced to colorless hydroresorufin. The latter reaction is reversible; therefore, in the medium containing thioglycollate the reduced portion is colorless, whereas the oxidized portion is pink. An attempt was made to use resorufin in place of resazurin but we obtained a brownish red color which was

more difficult to detect than the pink obtained from resazurin. There also was doubt about its chemical purity even though it was equally noninhibitory.

TABLE 1

Bacteriostatic action of Eh indicators

CULTURE	NO INDICATOR				METHYLENE BLUE				METHYLENE BLUE ZINC CHLORIDE 9781				METHYLENE BLUE ZINC CHLORIDE 140582				RESAZURIN			
	Day																			
	1	2	4	7	1	2	4	7	1	2	4	7	1	2	4	7	1	2	4	7
Mich. 6		3*	4	4														3	5	5
Mich. 14	2	4	4	4		2	3	3		1	3	3		1	2	3	2	3	4	4
Mich. 38		3	3	3			1	2			1	2			1	1		3	4	4
Mich. 48	2	3	5	5				1				1				1	1	1	4	4
Mich. 53		2	3	3														2	3	3
Mich. 54		1	2	2															2	2
Mich. 57		2	2	2				1				1				1	2	3	3	3
Mich. 60	2	4	5	5				1							1	2	2	4	4	4
Total numbers of tubes	6	22	28	28		2	4	8		1	4	7		1	4	8	7	19	29	29
Total turbidity values†	9	39	85	98		2	7	17		1	6	13		2	7	15	10	35	83	100

The formula of the medium differed from that of the revised in specifying 0.1 per cent of *l*-cystine and 0.5 per cent of sodium chloride. The methylene blue and resazurin were present in a final concentration of 1:500,000; the zinc salts were added to give a concentration of 1:500,000 of methylene blue.

* Numerals indicate the number of tubes inoculated with tenfold dilutions of the cultural suspension, which had visible turbidity.

† The degree of turbidity of growth was expressed as 1, 2, 3, and 4. The value given in the table is the summation of the values of the respective tubes.

TABLE 2

Influence of resazurin on growth of bacteria

NUMBER OF CULTURES	RESAZURIN															
	None				1:1,000,000				1:250,000				1:100,000			
	Day															
	1	2	4	7	1	2	4	7	1	2	4	7	1	2	4	7
Aerobic, 7	13.5	26.5	30.5	31.5	11.5	25.0	29.5	31.0	11.5	24.5	30.0	32.0	4.5	10.0	21.0	27.0
Anaerobic, 4	18.5	20.5	21.5	21.5	17.5	18.5	18.5	18.5	17.5	19.5	19.5	20.0	19.5	21.5	21.5	21.5
Total, 11	32.0	47.0	52.0	53.0	29.0	43.5	48.0	49.5	29.0	44.0	49.5	52.0	24.0	31.5	42.5	48.5

The formula of the medium differed from that of the revised in specifying 0.5 per cent of sodium chloride. Each dilution was inoculated into duplicate tubes of medium. The numerals represent the sum of the average of the duplicates which showed visible turbidity.

For several years the use of resazurin in a reduction test to determine the sanitary condition of milk has been gradually replacing the use of methylene blue

(Ramsdell, Johnson, and Evans, 1935; Davis, 1942; Johns, 1942). Because of the need of a pure product for this use, resazurin can now be obtained in a relatively pure state.

Peptone

Peptones derived from casein have been used exclusively in this investigation. Preliminary tests showed that the development of even the fastidious bacteria

TABLE 3

Comparison of pancreatic and acidic hydrolyzates of casein

NUMBER OF CULTURES	TYPE OF HYDROLYSIS	DEVELOPMENT OF BACTERIA—DAY							
		Number of tubes				Turbidity values			
		1	2	4	7	1	2	4	7
20 (Medium 1)	Pancreatin	57	81	89	89	149	240	321	337
	Acid	35	67	77	81	91	188	268	291
8 (Medium 2)	Pancreatin	17	35	38	38	44	91	135	150
	Acid	15	31	39	40	39	81	129	145
5 (Medium 3)	Pancreatin	9	20	27	27	22	46	81	97
	Acid	7	16	22	22	18	33	64	72
Totals: 33	Pancreatin	93	136	154	154	215	377	537	584
	Acid	57	114	138	143	148	202	461	508

Media formulae:

	NO. 1	NO. 2	NO. 3
	g per L	*g per L*	*g per L*
Casein hydrolyzate	10.0	10.0	10.0
Yeast extract	10.0	10.0	10.0
Glucose	5.0	5.0	5.0
Sodium chloride	5.0	2.5	5.0
Sodium thioglycollate	0.5	0.5	0.5
Agar	0.7	0.8	0.8
Dipotassium phosphate		1.0	
Resazurin	0.001	0.001	0.001

was promoted by these peptones. It is believed by some that a peptone prepared by the pancreatic digestion of milk protein is more uniform in composition than a similar product prepared as a by-product of the meat-packing industry. Nonallergenic peptone can be made without difficulty; this is of prime importance when antigens intended for human parenteral use contain culture medium. Furthermore, it lends itself to a detailed description so that the product can be duplicated at will (Leifson, 1943).

Enzyme versus acid hydrolyzate. Pancreatic or tryptic digests of casein have been found to be more desirable than an acid hydrolyzate (bacto-casamino acids).

The former contain a larger number of amino acids. Of particular note is the presence of tryptophane. The results of three experiments, in which the growth-promoting activity of peptones obtained by means of the two types of hydrolysis was compared, are given in table 3. In each experiment the initiation of growth was earlier in the presence of the pancreatic digest. However, by the end of 7 days the difference was reduced. Nevertheless, in the case of sterility testing when it is most desirable to detect contamination as early as possible, the use of the pancreatic digest would be indicated in preference to the acid hydrolyzate.

Comparison of different preparations of pancreatic digest of casein. The growth-promoting activity of pancreatic or tryptic digests prepared by 5 manufacturers has been studied. The results of 2 experiments are summarized in table 4. It is apparent that when the respective peptones were used in combination with the other ingredients of the medium, there was no significant difference in their promotion of the growth of the exacting bacteria which had been selected for

TABLE 4

Pancreatic digests of casein prepared by different manufacturers

NUMBER OF CULTURES	DESIGNATION OF PEPTONE	DEVELOPMENT OF BACTERIA—DAY							
		Number of tubes				Turbidity values			
		1	2	4	7	1	2	4	7
11*	A	18	32	42	42	45	87	144	161
	B	17	33	38	42	50	99	143	151
	C	18	29	37	40	44	91	131	147
	D	16	30	43	44	43	84	151	165
10†	A	23	39	45	48	44	107	152	167
	E	19	36	43	46	39	97	144	168

* The formula of the medium differed from that of the revised in specifying 0.1 per cent of *l*-cystine and 0.07 per cent of agar.

† Revised formula.

these experiments. However, in other experiments, in which the yeast extract was omitted from the medium, certain preparations were definitely better than others.

Yeast Extract

The addition of a water-soluble extract of yeast definitely favored the development of the bacteria. The results of 2 experiments are given in table 5. In the first the growth of bacteria in a medium containing 2.0 per cent of peptone was compared with that in a medium containing 1.0 per cent of peptone and 1.0 per cent of yeast extract. In the presence of the latter there was growth at the end of 24 hours in 10 more tubes, or 21 per cent, than in the former; and at the end of 48 hours the difference had increased to 20 tubes, or 33 per cent.

In the other experiment 3 concentrations of yeast extract, 0.2, 0.5, and 1.0 per cent, were used in combination with 2.0, 1.5, and 1.0 per cent of the peptone,

respectively. The best development was obtained in the presence of 0.5 per cent yeast extract and 1.5 per cent peptone. These results are not significantly different from those obtained with the medium containing 1.0 per cent of yeast extract and 1.0 per cent of peptone. It is definite, however, that in the presence of 0.2 per cent of the yeast extract and 2.0 per cent of peptone there was a lag in the initiation of growth. We have observed no inhibition in development in the presence of 1.0 per cent of the yeast extract, but Dr. G. B. Slocum (personal communication) has noted that *Lactobacillus* is inhibited in the presence of more than 0.5 per cent. It appeared that 0.5 per cent of yeast extract would be an adequate and satisfactory amount to use in the fluid thioglycollate medium. No doubt the extract supplies certain growth factors which aid in the promotion of the development of bacteria. Leifson (1943) has reported that the addition of

TABLE 5

Influence of varying amount of yeast extract in the presence of different amounts of peptone

NUMBER OF CULTURES	VARIATIONS IN MEDIA		DEVELOPMENT OF BACTERIA—DAY							
	Peptone	Yeast ext.	Number of tubes				Turbidity values			
			1	2	4	7	1	2	4	7
	%	%								
20	2.0	0.0	47	61	73	74	131	176	251	270
	1.0	1.0	57	81	89	89	149	240	321	337
21	2.0	0.2	34	64	77	81	110	193	246	276
	1.5	0.5	44	72	80	83	121	214	267	300
	1.0	1.0	44	67	77	80	121	211	267	295

Formula of basic medium: For amounts of casein peptone and yeast extract see above; glucose, 0.5%; sodium chloride, 0.5%; *l*-cystine, 0.1%; sodium thioglycollate, 0.05%; agar, 0.07%; and resazurin, 0.0001%.

accessory growth factors greatly improve the general nutritive properties of casein peptones.

Buffer

It is shown in table 6 that more rapid development of the bacteria took place in the absence of the buffer (K_2HPO_4) than in its presence. At the end of 24 and 48 hours there was growth in 10 (29 per cent) and 16 (31 per cent) more tubes, respectively. However, at the end of 7 days there was growth in the same number of tubes of each medium. The effect of the buffer seemed to be retardation. In this experiment the buffer was added to adjust the pH, and 0.3 per cent was required. This amount is slightly in excess of the 0.25 per cent that was specified in the first formulae of the fluid thioglycollate media recommended by the National Institute of Health. It may be that this additional amount of the potassium ion acted as an inhibitor. Since the amount of buffer originally used is not sufficient to neutralize the acidity that might be produced from the

0.5 per cent glucose, and its presence is not needed for the development of the bacteria, it seemed better to omit it from the formula.

Sodium Chloride and Hydrogen Ion Concentration

Bacteria of different species vary in their requirements for sodium chloride and hydrogen ion concentration in order to attain optimum development. In table 7 it is shown that *Clostridium acetobutylicum*, 824, developed best in the medium with the lowest pH used, 7.1, to which no sodium chloride had been added. The pH of the medium was more influential than the amount of sodium chloride. This is graphically illustrated in figure 1. Here it may be seen that this culture developed in the presence of 0.5 per cent of sodium chloride at a pH of 7.11, although there was some retardation. On the other hand, in the

TABLE 6

Influence of buffer, dipotassium phosphate

NUMBER OF CULTURES	BUFFER K_2HPO_4	DEVELOPMENT OF THE BACTERIA—DAY							
		Number of tubes				Turbidity values			
		1	2	4	7	1	2	4	7
	%								
21	0.3	34	51	72	80	100	169	223	276
	None	44	67	77	80	121	211	267	295

Formula of basic medium: Casein peptone, 1.0%; yeast extract, 1.0%; glucose, 0.5%; sodium chloride, 0.5%; *l*-cystine, 0.1%; sodium thioglycollate 0.05%; agar 0.07%; and resazurin 0.0001%.

medium with a pH of 7.35, to which no sodium chloride had been added, there was very little development. The Yale strain of *C. acetobutylicum* was not so sensitive to variations in pH.

In contrast it is shown, also in table 7, that the Montana strain of *Clostridium chauvoei* developed better in the presence of sodium chloride than in its absence, although there was a tendency for it to favor the low pH. This preference may be observed best by comparing the turbidity of growth at the end of 1 and 2 days in the presence of the different salt concentrations at the different pH values. The other strain of *C. chauvoei* studied behaved similarly.

Differing from the requirements of both *C. acetobutylicum* and *C. chauvoei* were those of the type 3 pneumococcus (table 7). It developed best in the presence of the highest salt concentration and highest pH that were employed, 0.5 per cent and 7.32, respectively. Other strains of the pneumococcus were not so sensitive to variations. However, with the fluid thioglycollate medium the optimum pH for the pneumococcus seemed to be between 7.2 and 7.3 and the presence of sodium chloride was indicated as desirable.

In another experiment it was observed that the *Micrococcus*, Mich. 38, and the *Micromonospora*, Mich. 53, developed better at pH 7.4 than at 7.2 or 7.0. The presence or absence of sodium chloride did not appear to be significant.

In order to promote the development of bacteria which vary in their requirements for sodium chloride and hydrogen ions, a mid-course was followed and a concentration of 0.25 per cent sodium chloride and a pH of 7.1 ± 0.1 were

TABLE 7

The influence of sodium chloride in the presence of varying hydrogen ion concentrations

CULTURE	SODIUM CHLORIDE	pH	DEVELOPMENT OF THE BACTERIA—DAY							
			Number of tubes				Turbidity values			
			1	2	4	7	1	2	4	7
	%									
C. acetobutylicum, 824	0.5	7.32								
		7.11		3	4	4		11	16	16
		7.0	3	4	4	4	7	16	16	16
	0.25	7.32								
		7.11	3	4	4	4	6	16	16	16
		7.0	4	4	4	4	13	16	16	16
		7.35		1	1	1		4	4	4
		7.18	4	4	4	4	11	16	16	16
		7.1	5	5	5	5	15	20	20	20
C. chauvoei, Mont.	0.5	7.32	3	4	4	4	3	11	16	16
		7.11	3	3	3	3	5	11	12	12
		7.0	3	3	3	3	9	11	12	12
	0.25	7.32	2	3	3	3	2	4	11	12
		7.11	2	3	4	4	2	8	15	16
		7.0	4	4	4	4	7	16	16	16
		7.35		1	3	3		1	9	12
		7.18	1	2	3	3	1	3	8	12
		7.1	1	1	5	5	1	3	10	20
Diplococcus pneumoniae, type 3	0.5	7.32	4	4	5	5	7	12	17	20
		7.11	3	5	5	5	5	13	20	20
		7.0	3	5	5	5	4	5	17	20
	0.25	7.32	3	3	4	4	3	3	16	16
		7.11	3	3	4	4	4	8	13	16
		7.0	2	4	4	5	2	8	16	16
		7.35		1	3	3		2	7	12
		7.18	1	1	3	3	1	2	10	12
		7.1	1	1	3	3	1	3	9	12

Revised formula.

selected for the medium. It should be mentioned that the ingredients used in the preparation of the medium must have a uniformly low sodium chloride content.

Besides the bacterial need for sodium chloride, the presence of the salt in the sterility test medium may be necessary for the development of contaminants in some biological products containing mercurial preservatives. In one of the early experiments when many variations were being studied, 14 different media were

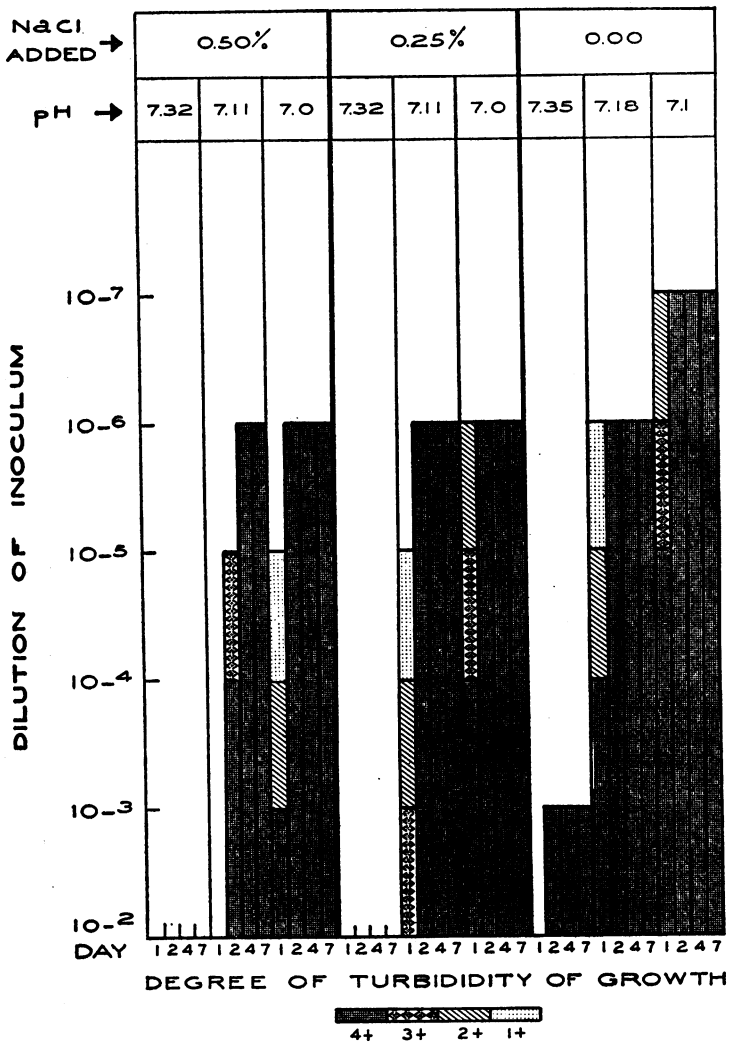

FIG. 1. THE GROWTH OF C. ACETOBUTYLICUM, STRAIN 824, IN THE PRESENCE OF VARYING AMOUNTS OF SODIUM CHLORIDE AND DIFFERENT pH CONCENTRATIONS

under test. Several contaminated products were seeded into the media. With a normal serum albumin solution containing 1:10,000 merthiolate, it was observed that in 7 of the media no growth developed from the inocula of the undiluted and the 10^{-1} dilution, but excellent growth developed from the 10^{-2}, 10^{-3}, and 10^{-4} dilutions. In the other 7 media there was development from all 5 inocula.

The media differed considerably in composition. The only common factor in the first seven was that no sodium chloride had been added. Salt had been added to the other media although only 0.15 per cent had been added to two. It is thought that perhaps the salt combined with the mercurial compound to form a less soluble product, thereby permitting the bacteria to grow. The contaminant was a diphtheroid. Dilutions of a suspension of this culture from agar initiated growth in all 14 media.

The presence of sodium chloride was not the controlling factor in the development of the bacteria in the other biological products tested at the same time.

l-Cystine

C. chauvoei does not develop in fluid thioglycollate medium without the addition of *l*-cystine. The chances for this organism to contaminate a biological

TABLE 8

Influence of varying amounts of l-cystine on the development of C. chauvoei Mont.

DILUTION OF INOCULUM	AMOUNT OF *l*-CYSTINE—PER CENT														
	0.1			0.075			0.05			0.025			None		
	Day														
	2	4	7	2	4	7	2	4	7	2	4	7	2	4	7
10^{-2}	4	4	4	4	4	4	3	4	4	3	4	4			3
10^{-3}	4	4	4	4	4	4	4	4	4	1	2	4			
10^{-4}	4	4	4	4	4	4	2	4	4	2	3	4			
10^{-5}	4	4	4	4	4	4	3	4	4		3	4			
10^{-6}	4	4	4	4	4	4		4	4		3	4			
10^{-7}															

The medium was prepared according to the revised formula with variations in the amount of cystine. Numerals indicate degree of turbidity.

product prepared for human use are remote. On the other hand, if the medium is to be used in connection with products for veterinary use, it would be necessary for it to be adequate for the development of this organism. So far as we have been able to determine, there is no contraindication to its inclusion and it is probable that its presence may stimulate the development of other bacteria.

From the results of the experiment reported in table 8 it appears that 0.075 per cent of *l*-cystine is sufficient to obtain the most rapid growth of *C. chauvoei;* 0.05 per cent would probably be sufficient, but with less than 0.05 per cent there is a lag. Note how this organism failed to grow in the medium containing no *l*-cystine.

Shortly after the official date of adoption of the revised formula (January 15, 1945) Hickey suggested the inclusion of cysteine hydrochloride in fluid thioglycollate medium for the sterility testing of penicillin. Since *l*-cystine had been included in the medium, it was thought that perhaps part of the *l*-cystine would be reduced to cysteine in the presence of the thioglycollate and that the new

medium might be suitable for the sterility testing of penicillin without the addition of an inactivating agent. Hewitt and Pittman (1945) found, however, that there was no inactivation of penicillin in the medium in a ratio of 10 units to 0.75 mg of *l*-cystine within 3 hours.

Temperature for Incubation

Sufficient experimentation has not been done to determine the optimum temperature for incubation of the sterility test. However, so far as the work has gone, it is indicated that 32 C might be favorable to all contaminants. One experiment was conducted in duplicate with incubation at 36 C and at 22 to 25 C. All of the cultures grew at the lower temperature, whereas one did not grow, and others grew more poorly, at the higher temperature. Miss Frances Clapp, of Lederle Laboratories, has reported to us the isolation of contaminants with incubation at 31 C when no growth was obtained with incubation at 37 C. On the other hand, she has obtained no growth at 37 C that she has not likewise obtained at 31 C.

So far as we know there are no bacteria that might occur as contaminants that develop at 37 C and will not likewise develop at 31 or 32 C, whereas there are a number which will not grow at 37 C. In the latter group there are some of the *Pseudomonas* cultures, and these are notorious for their ability to be pyrogenic (Probey and Pittman, 1945).

SUMMARY

A study has been presented which formed the basis for the adoption of a revised formula for the "Fluid thioglycollate medium"; this formula replaces the two previously recommended for use in the testing of biological products for sterility. It was desirable that all ingredients included in the formula should lend themselves to full description. Such descriptions in addition to full directions for preparing the medium and its use are given in the National Institute of Health circular, "Culture Media for the Sterility Test," dated January 15, 1945, and it is anticipated that they will be included in the thirteenth edition of the *U. S. Pharmacopoeia*.

Resazurin was found to be a suitable Eh indicator. In concentrations of 1:250,000 or less, resazurin did not retard the development of the bacteria tested, whereas methylene blue in a concentration of 1:500,000 was definitely bacteriostatic for certain bacteria. A 1:1,000,000 dilution of the resazurin is sufficient to indicate oxidation-reduction conditions.

Pancreatic digest of casein is apparently an adequate peptone for the sterility test medium. It has excellent growth-promoting properties which should make it useful in many kinds of media. In its presence, initiation of growth was more rapid than in the presence of an acid hydrolyzate of casein.

A water-soluble extract of yeast was influential in promoting growth of the bacteria. The optimum amounts of the extract and the casein peptone appear to be 0.5 and 1.5 per cent, respectively.

Dipotassium phosphate tended to retard initiation of growth.

A pH of 7.1 and the addition of 0.25 per cent of sodium chloride seem to provide suitable conditions for the development of those bacteria that prefer a low pH and no sodium chloride and those that prefer a higher pH and 0.5 per cent of sodium chloride.

The addition of *l*-cystine enables *Clostridium chauvoei* to develop in the medium.

The optimum temperature for incubation of the sterility test is discussed.

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