CULTIVATION OF BACTERIUM TULARENSE IN SIMPLIFIED LIQUID MEDIA

JOSEPH T. TAMURA AND IRVIN W. GIBBY

Department of Bacteriology, College of Medicine, University of Cincinnati, and the Cincinnati General Hospital

Received for publication September 8, 1942

Since the isolation and cultivation of *Bacterium tularense* by McCoy and Chapin in 1912, and the demonstration of its obligate cystine requirement by Francis in 1922, little has been written concerning its nutritional requirements.

Lufkin and Evenson (1936–7) reported "abundant growth" of *Bacterium tularense* in a "routine" blood culture in glucose beef infusion broth. It is clear that the organism survived for more than eleven days in this medium, but their statement that the culture continued to grow well in successive subcultures in glucose broth is not supported by convincing evidence. They also stated that "growth" had been obtained under similar cultural conditions by Gundry and Warner (1934), but that paper presents no evidence for multiplication; only for survival for a period of three days.

Brigham and Rettger (1935) obtained growth in blood and serum infusion broths in which cultures were incubated in air to which had been added 25 per cent of a mixture of 95 per cent oxygen and 5 per cent carbon-dioxide. The inocula were heavy. Serial transfer in these media showed growth to be uncertain in the absence of added oxygen and carbon-dioxide or cystine.

Berkman and Koser (1941) reported growth in a basal medium of hydrolyzed gelatin to which thiamin or thiamin diphosphate was added. Large inocula were necessary and serial subcultures were not described.

In a recent study of fermentation Francis (1942) stated, "No liquid medium is suited to its growth." For this work he used serum cystine infusion agar.

We have studied the nutritional requirements of *Bacterium tularense*, including a number of substances which influence its growth. Since definition of all factors essential for growth may well prove to be a time-consuming problem, it seems appropriate to record several simplified liquid media, without peptone, which will support sustained growth of the organism and in which continued serial transfer is possible without loss of initial virulence.

MATERIALS AND METHODS

Three basal media were used in this investigation. The components were selected arbitrarily and their concentrations were varied until a combination was found which supported excellent growth in the presence of the proper accessory growth factors. The formulae of the basal media without accessory growth factors consisted of:

1. Synthetic basal medium

Most of the amino acids are found in gelatin, and were used in amounts roughly proportional to their occurrence in this protein.

Amino acids	*Concentration in grams/liter*
Glycine	1.20
l (+) glutamic	0.40
d-arginine	0.20
l-proline	0.35
d-lysine	0.25
l-histidine	0.20
dl-alanine	0.50
dl-phenylalanine	0.10
dl-leucine	0.30
l-tryptophane	0.05
dl-valine	0.10
dl-serine	0.015
asparagine	0.15
l-cystine	0.10
Salts	
NaCl	8.50
$Na_2HPO_4 \cdot 12H_2O$	0.50
KH_2PO_4	0.25
$MgSO_4$	0.05
$FeSO_4 \cdot 7H_2O$	0.00074
Glucose	5.00

2. Hydrolyzed gelatin basal medium

Fischer's gold label gelatin was hydrolyzed with hydrochloric acid according to the method of Williams (1941). The final concentration of chloride ion was equivalent to 3.81 per cent sodium chloride. Nitrogen content was determined by Micro-Kjeldahl.

The medium was composed as follows:

l-cystine	0.10 gm.
glycerol	5.00 gm.
NaCl (total)	10.00 gm.
$Na_2HPO_4 \cdot 12H_2O$	0.40 gm.
KH_2PO_4	0.20 gm.
$MgSO_4$	0.04 gm.
$FeSO_4 \cdot 7H_2O$	0.00074 gm.
Gelatin acid hydrolysate, 0.28% total N	1000 ml.

3. Hydrolyzed casein basal medium

Casein hydrolysate was prepared from SMA vitamin-free casein in the same manner. Other constituents were essentially the same as those of gelatin hydrolysate medium.

Cystine was first dissolved in one per cent hydrochloric acid and added before adjusting the pH. The media were adjusted to pH 6.9, tubed in 4.5 ml. quantities in 25 mm. diameter tubes and autoclaved at 10 lbs. for 10 minutes.

Cultures. The Memp strain of *Bacterium tularense* was used throughout the study unless otherwise stated. This strain was isolated from a human lymph node in 1937, and had been passed through guinea pigs since isolation. It was highly virulent.

Method of inoculation. Bacterial cells from 24- to 48-hour cultures, grown either in gelatin hydrolysate medium or on cystine-heart-hemoglobin agar, were washed with sterile saline three times and resuspended to a turbidity of T-500 (Fullers' earth scale). From this suspension serial 1:10 dilutions were prepared. Hereafter the T-500 suspension is designated as 10° and its dilutions are designated in the usual manner. One half ml. of the desired suspension was then used to inoculate each tube. Accurate plate counts of suspensions cannot be made, but Foshay has estimated from numerous animal inoculations that the number of viable bacterial cells in one ml. of a T-500 suspension is approximately two billions.

Growth. As most of the media used were water clear and practically colorless, growth was detected by the appearance of turbidity in the cultures.

EXPERIMENTAL

1. Synthetic medium

A number of accessory growth substances were tested for their effect on growth. The substances tested, and the ranges of concentrations, were as follows:

	micrograms/ml.
thiamin	1.0 to 50.0
riboflavin	0.1 to 5.0
pimelic acid	0.1 to 2.0
calcium d-pantothenate	1.0 to 4.0
nicotinic acid	0.1 to 1.0
nicotinamide	0.1 to 1.0
beta-alanine	1.0 to 4.0
choline	1.0 to 10.0
inositol	1.0 to 20.0
uracil	10.0 to 50.0
adenine sulfate	10.0 to 50.0
guanine hydrochloride	10.0 to 50.0
biotin concentrate (SMA)	0.01 to 0.1

	dilutions in medium
biotin concentrate*	1:150 to 1:500
liver extract†	0.5% to 0.05%
liver cake aqueous extract‡	1:150 to 1:500
blood cell extract§	0.1% to 1.0%

* This biotin concentrate was prepared by hydrolyzing liver-cake in N/1 H_2SO_4 for 4 to 5 hours at 120° to 125° C. so that one ml. of the hydrolysate represents the equivalent of 0.2 gm. of liver-cake.

† Mother-liquor from alcohol precipitation of crude liver extracts. One gm. of the extract equals 300 gms. of fresh liver.

The synthetic basal medium without addition of accessory factors did not support growth. Growth was obtained by addition of:

	micrograms/ml.
calcium d-pantothenate	4.0
pimelic acid	1.0
nicotinic acid	0.5
biotin concentrate	1:150

Organisms grown in this medium were viable for at least 14 days of continued incubation as judged by subcultivation onto standard solid media.

Table 1 indicates good maintenance of virulence by the organism after prolonged continuous cultivation in this medium.

TABLE 1

Titration of virulence in mice of Bacterium tularense cultivated in synthetic medium

NUMBER OF MICE USED	DILUTION OF CULTURE INJECTED	AMOUNT INJECTED	DEATH OF ANIMALS IN DAYS							SURVIVALS	REMARKS
			1	2	3	4	5	6	7		
		ml.									
10	10^{-6}	0.5	0	0	8	2				0	43 day culture with
10	10^{-7}	0.5	0	0	3	7				0	total of 13 subcul-
10	10^{-8}	0.5	0	0	0	7	2	1		0	tures
10	10^{-9}	0.5	0	0	1	2	3	2		2	
10	10^{-6}	0.5	0	0	2	8				0	100 day culture with
10	10^{-7}	0.5	0	0	0	9	1			0	total of 31 subcul-
10	10^{-8}	0.5	0	0	0	9	1			0	tures
10	10^{-9}	0.5	0	0	0	2	7			1	

Animals were injected intraperitoneally.

The culture medium was centrifuged and the bacterial cells were resuspended to T-500, from which serial 1:10 dilutions were made in sterile saline.

Replacement of biotin concentrate in synthetic medium. In the mixture of amino acids, salts, glucose, pantothenic acid, pimelic acid, and nicotinic acid, the biotin concentrate remained the only chemically undefined constituent of the medium. The substitution of biotin methyl ester for biotin concentrate was not successful, though a trace of growth was initiated after 5 days of incubation. Adenine sulfate, guanine hydrochloride, uracil, etc., were supplemented

‡ Two hundred gms. of liver-cake was infused in 1 liter of distilled water over-night in refrigerator. It was autoclaved at 5 lbs. for 10 minutes and filtered through paper after cooling. One ml. of the extract equals 0.2 gms. of liver-cake.

§ Defibrinated fresh rabbit blood cells were washed five times with sterile saline. Packed cells were hemolyzed in 20 volumes of sterile distilled water. Heat coagulable protein was removed by filtration through paper after boiling in a water bath for five minutes. The filtrate was again boiled for 10 minutes. Slightly turbid solution was cleared by centrifugation.

With the exception of thiamin, all were prepared in aqueous solution and added in desired amounts to the basal medium before autoclaving.

with biotin methyl ester to substitute for biotin concentrate without demonstrable success. This indicated the presence of a chemically undefined substance or substances in the biotin concentrate which could not be totally replaced by any combination of accessory factors employed. The results of various combinations studied are shown in table 2.

2. Gelatin hydrolysate medium

The results of a typical growth experiment with hydrolyzed gelatin medium supplemented with various accessory factors are shown in table 3. Blood-cell extract, liver-cake fractions, and biotin concentrates were effective in pro-

TABLE 2

Effect of various growth factors on Bacterium tularense in a synthetic medium

BASAL MEDIUM (SALTS, 14 AMINO ACIDS, GLUCOSE) PLUS:	GROWTH DURING A 7 DAY INCUBATION PERIOD
1. Nothing	0
2. Pantothenic acid	0
3. Pimelic acid	0*
4. Nicotinic acid	0
5. Biotin, methyl ester	0*
6. Pantothenic acid, nicotinic acid, pimelic acid	0
7. Pantothenic acid, pimelic acid, nicotinic acid, riboflavin	0
8. Pantothenic acid, pimelic acid, nicotinic acid, choline	0
9. Pantothenic acid, pimelic acid, nicotinic acid, riboflavin, thiamin	0
10. Pantothenic acid, pimelic acid, nicotinic acid, biotin methyl ester	± to +
11. Pantothenic acid, pimelic acid, nicotinic acid, adenine sulfate, guanine hydrochloride, uracil, biotin methyl ester	+
12. Pantothenic acid, pimelic acid, biotin concentrate	++++
13. Biotin concentrate	+++
14. Pimelic acid, biotin methyl ester	±

All tubes were inoculated with 0.5 ml. of a 10^{-3} dilution of a T-500 suspension and incubated at 37°C. for 7 days when the final readings were made.

0, no visible turbidity; ±, trace of turbidity; +, very light turbidity; ++, light to moderate turbidity; +++, moderate to heavy turbidity; ++++, heavy turbidity.

* Occasionally a trace of turbidity appeared.

moting growth. In the presence of any one of these materials growth was obtained from very small inocula. Visible growth was not obtained in the basal medium alone during 7 days of incubation if the inocula contained fewer than 1,000,000 organisms. If inocula were larger than this, scanty growth was obtained. Addition of pantothenic acid, nicotinic acid, inositol, thiamin, and riboflavin, singly and in various combinations, resulted in no appreciable improvement of growth.

Prolonged dialysis or adsorption on vegetable charcoal removed the growth-stimulating substance from blood cell extract.

In basal media without accessory factors, gelatin hydrolysate supported very light growth, whereas in the synthetic medium no growth was initiated.

TABLE 3

Effect of various growth factors on Bacterium tularense in hydrolyzed gelatin medium

INOCULUM 10:	Days of incubation						
BASAL MEDIUM PLUS: Nothing	1	2	3	4	5	6	7
-1	+	+	++	++	++	+++	+++
-2	0	0	±	+	+	+	++
-3	0	0	0	0	0	0	0
-4	0	0	0	0	0	0	0
-5	0	0	0	0	0	0	0
-6	0	0	0	0	0	0	0
-7	0	0	0	0	0	0	0
-8	0	0	0	0	0	0	0
-9	0	0	0	0	0	0	0
BASAL MEDIUM PLUS: Pan., pim., nic., bio.							
-1	+	+	++	+++	+++	++++	++++
-2	0	+	+	++	+++	+++	++++
-3	0	0	+	+	++	++	++++
-4	0	0	0	+	+	+++	+++
-5	0	0	0	0	0	±	±
-6	0	0	0	0	0	0	0
-7	0	0	0	0	0	0	0
-8	0	0	0	0	0	0	0
-9	0	0	0	0	0	0	0
Pan., pim., nic., bio. con.							
-1	++	+++	+++	++++	++++	++++	++++
-2	+	++	+++	++++	++++	++++	++++
-3	0	+	+++	++++	++++	++++	++++
-4	0	0	+	++	+++	++++	++++
-5	0	0	0	+	++	+++	++++
-6	0	0	0	0	0	+	+++
-7	0	0	0	0	0	0	+
-8	0	0	0	0	0	0	0
-9	0	0	0	0	0	0	0
Pan., pim., nic., BC ex.							
-1	++	+++	+++	++++	++++	++++	++++
-2	+	++	+++	++++	++++	++++	++++
-3	0	+	++	+++	++++	++++	++++
-4	0	+	++	+++	++++	++++	++++
-5	0	0	+	++	+++	++++	++++
-6	0	0	0	+	+	+++	++++
-7	0	0	0	0	+	+	+++
-8	0	0	0	0	±	+	++
-9	0	0	0	0	0	0	0
Pan., pim., nic., LC ex.							
-1	++	+++	++++	++++	++++	++++	++++
-2	++	+++	++++	++++	++++	++++	++++
-3	+	+++	++++	++++	++++	++++	++++
-4	0	+	+++	++++	++++	++++	++++
-5	0	+	++	+++	++++	++++	++++
-6	0	0	+	++	+++	++++	++++
-7	0	0	±	+	+++	++++	++++
-8	0	0	0	+	++	+++	++++
-9	0	0	0	0	±	+	+++
Pan., pim., nic., inos., bio.							
-1	+	+	++	+++	+++	++++	++++
-2	0	+	+	++	+++	+++	++++
-3	0	0	+	++	++	+++	++++
-4	0	0	+	+	++	+++	+++
-5	0	0	0	+	+	++	+++
-6	0	0	0	0	0	0	±
-7	0	0	0	0	0	0	0
-8	0	0	0	0	0	0	0
-9	0	0	0	0	0	0	0
LC ex.							
-1	++	+++	++++	++++	++++	++++	++++
-2	++	+++	++++	++++	++++	++++	++++
-3	+	++	+++	++++	++++	++++	++++
-4	0	+	+++	++++	++++	++++	++++
-5	0	+	++	+++	++++	++++	++++
-6	0	0	+	++	++++	++++	++++
-7	0	0	+	+	++	+++	++++
-8	0	0	0	+	++	+++	++++
-9	0	0	0	0	±	++	+++
BC ex.							
-1	++	+++	+++	++++	++++	++++	++++
-2	+	++	+++	+++	++++	++++	++++
-3	0	+	++	+++	+++	++++	++++
-4	0	0	+	++	+++	++++	++++
-5	0	0	±	+	++	+++	++++
-6	0	0	0	±	±	+	++
-7	0	0	0	0	±	+	++
-8	0	0	0	0	0	0	+
-9	0	0	0	0	0	0	0
Pan., pim., nic., dialyzed BC ex.							
-1	+	+	++	++	++	+++	+++
-2	0	0	+	+	+	+	++
-3	0	0	0	0	0	0	0
-4	0	0	0	0	0	0	0
-5	0	0	0	0	0	0	0
-6	0	0	0	0	0	0	0
-7	0	0	0	0	0	0	0
-8	0	0	0	0	0	0	0
-9	0	0	0	0	0	0	0
Pan., pim., nic., charcoal filtrate of BC ex.							
-1	+	+	++	++	++	+++	+++
-2	0	0	+	+	+	+	++
-3	0	0	0	0	0	0	0
-4	0	0	0	0	0	0	0
-5	0	0	0	0	0	0	0
-6	0	0	0	0	0	0	0
-7	0	0	0	0	0	0	0
-8	0	0	0	0	0	0	0
-9	0	0	0	0	0	0	0

Pan, calcium d-pantothenate, 4 μg./ml.; pim, pimelic acid, 1 μg./ml.; nic, nicotinic acid, 0.5 μg./ml. bio. biotin (SMA) 0.02 μg./ml.; inos. inositol, 20 μg./ml.; BC ex. blood cell extract, 1%; bio. con. biotin concentrate, 1:150; LC ex. liver-cake aqueous extract, 1:250.

0, no visible turbidity; ±, trace of turbidity; +, very light turbidity; ++, light to moderate turbidity; +++, moderate to heavy turbidity; ++++, heavy turbidity.

It is evident that hydrolyzed gelatin contains a substance or substances which will support limited growth.

Effect of addition of certain amino acids. To a liter of gelatin hydrolysate medium was added a supplement of 6 amino acids; 30 mgs. each of l-tryptophane, dl-valine, dl-leucine, dl-methionine; 15 mgs. of l-histidine and 10 mg. of dl-threonine. In this medium growth was apparently accelerated during the first 3 days of incubation. However, no quantitative difference could be observed at the end of 7 days of incubation in comparison with hydrolyzed gelatin medium without addition of amino acids.

3. Hydrolyzed casein medium

With vitamin-free casein hydrolysate in place of gelatin, a basal medium was prepared in the same manner. When this medium was supplemented with blood cell extract or with liver-cake extract excellent growth was obtained. The amount of growth so obtained was essentially the same as that in gelatin hydrolysate medium.

Substitution of other sulfur-containing compounds. Homocystine, methionine, glutathione, and thioglycollic acid were used to replace cystine in gelatin hydrolysate medium. These substances were added to the medium in concentrations to equal the molecular equivalents of 0.01%, 0.05%, and 0.1% cystine. From 0.005% to 0.01% cystine is sufficient for excellent growth.

As shown in table 4, none of the compounds except glutathione and cystine were capable of supporting growth. The highest concentration of glutathione used was ten times the optimal molecular equivalent of cystine. Growth obtained in this concentration was scanty.

The present study of cultural requirements confirms the indispensability of cystine for growth.

Effect of various amounts of sodium chloride. For this purpose the following basic medium was used:

$Na_2HPO_4 \cdot 12H_2O$	0.4 gm.
KH_2PO_4	0.2 gm.
$MgSO_4$	0.02 gm.
$FeSO_4 \cdot 7H_2O$	0.00074 gm.
Cystine	0.1 gm.
Glycerol	5.0 gm.
Liver-cake extract	1:250
Pantothenic acid	0.004 gm.
Pimelic acid	0.001 gm.
Nicotinic acid	0.005 gm.
Gelatin hydrolysate free of chloride ion, 0.28% total N	1000 ml.

Sodium chloride was added to this medium in amounts from 0.25 to 2.0 per cent. Since sodium chloride resulted from adjustment of the medium to pH 6.9, the actual concentration was slightly greater than that indicated for each lot.

The presence of certain concentrations of sodium chloride in the basal medium proved important. The optimal concentration appeared to be one per cent or above. Two per cent was not inhibitory. The results of varying concentration of sodium chloride upon growth of *B. tularense* are shown in table 5.

TABLE 4

Growth in gelatin hydrolysate medium in which various compounds were substituted for cystine

BASIC MEDIUM (GELATIN HYDROLYSATE, SALTS, ACCESSORY FACTORS) PLUS: (TO EQUAL THE MOLECULAR EQUIVALENT OF CYSTINE IN %)		GROWTH OBSERVED AFTER 6 DAYS OF INCUBATION
No additions		0
Homocystine	0.01	0
	0.05	0
	0.1	0
Methionine	0.01	0
	0.05	0
	0.1	0
Glutathione	0.01	0
	0.05	0
	0.1	+
Thioglycollic acid	0.01	0
	0.05	0
	0.1	0
Cystine	0.0005	+
	0.001	+++
	0.005	++++
	0.01	++++
	0.05	++++

All tubes were inoculated with 0.5 ml. of a 10^{-3} dilution of a T-500 suspension.

TABLE 5

Effect of varying concentrations of NaCl upon growth of B. tularense

INOCULUM 10:	MEDIUM PLUS: NaCl %																										
	0.25%							0.5%							1.0%							2.0%						
	Growth in days																										
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
-2	0	±	+	+	+	+	+	0	±	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+	+	+	+
-3	0	0	0	+	+	+	+	0	0	±	+	+	+	+	0	+	+	+	+	+	+	0	±	+	+	+	+	+
-4	0	0	0	+	+	+	+	0	0	0	+	+	+	+	0	+	+	+	+	+	+	0	0	+	+	+	+	+
-5	0	0	0	0	±	±	+	0	0	0	+	+	+	+	0	0	+	+	+	+	+	0	0	+	+	+	+	+
-6	0	0	0	0	±	±	±	0	0	0	±	±	+	+	0	0	+	+	+	+	+	0	0	0	+	+	+	+
-7	0	0	0	0	0	±	±	0	0	0	0	±	+	+	0	0	0	+	+	+	+	0	0	0	+	+	+	+
-8	0	0	0	0	0	±	±	0	0	0	0	±	+	+	0	0	0	±	+	+	+	0	0	0	±	+	+	+
-9	0	0	0	0	0	0	0	0	0	0	0	0	0	±*	0	0	0	0	±	+	+	0	0	0	0	0	±	+

* Heavier growth appeared later.

± Trace of turbidity; +, pronounced turbidity.

Alkalinization of culture media. Attempts were made to determine fermentations of various carbohydrates with 20 strains of *B. tularense* in gelatin hydrolysate medium. Brom-cresol-purple or Andrade's solution were used as indicators. After carefully adjusting the medium to pH 6.9, the desired indicator was added in the usual amount. The medium was sterilized in 5 ml. quantity in tubes. The carbohydrates were previously sterilized and added to give a final concentration of 0.5%. Each tube was inoculated with 0.1 ml. of 10^{-2} saline suspension of 24 hour cultures grown on cystine-heart-hemoglobin agar.

No acid production was observed during 10 days of incubation from any of the 18 sugars tested. In all instances the reaction changed definitely to increased alkalinity.

TABLE 6

Alkalinization of culture media

INCUBATION	GELATIN HYDROLYSATE MEDIUM PLUS:			
	Nothing	0.5% glycerol	0.5% glucose	Uninoculated control
	pH changes from 6.9 to:			
days				
1	7.15	7.0	7.0	6.9
2	7.3	7.1	7.1	6.9
3	7.5	7.2	7.2	6.9
4	7.6	7.3	7.3	6.9
5	7.75	7.3	7.3	6.9
6	7.8	7.4	7.3	6.9
7	7.3*	7.4*	7.3*	6.9
8	8.0	7.4	7.3	6.9
9	8.0+	7.5	7.4	6.9
10	8.1†	7.5*	7.4*	6.9
11	8.1	7.6	7.4	6.9
12	8.1	7.7	7.4	6.9
13	8.1	7.7	7.5	6.9
14	8.1	7.7†	7.5*	6.9

* Organisms still viable.

† Organisms not viable.

Experiments were conducted to verify actual alkali production in gelatin hydrolysate medium. After careful adjustment to pH 6.9, two liters of medium were divided into four equal parts in liter flasks. To two flasks were added 0.5% glucose and 0.5% glycerol, respectively. The remaining two received no carbohydrate. Three flasks of media were inoculated with 2 ml. of a T-500 saline suspension of a 24-hour culture of the Memp strain. One flask was kept as an uninoculated control. Viability of the organism in the liquid media was tested from time to time by subcultivation on cystine-heart-hemoglobin agar during the period of incubation. As shown in table 6, alkali increased regularly from day to day, reaching a maximum in about 10 days in the medium without fermentable substance.

Toxin production. Three liter flasks, containing 300 ml. each of "complete" gelatin hydrolysate medium supplemented with liver-cake extract, were inoculated with 1.5 ml. of a heavy saline suspension of a 24-hour culture. After intervals of 5, 10, and 14 days of incubation these cultures were filtered through a Berkefeld N filter. Groups of 6 mice and 6 guinea pigs for each sample of filtrate were injected intraperitoneally with varying amounts of the filtrate ranging from 0.5 to 2 ml. for mice, and 1.0 to 5.0 ml. for guinea pigs.

All animals injected with the filtrates survived. Evidently the strain used did not produce a soluble toxin, or the hydrolyzed gelatin medium may not be suitable for toxin production.

Virulence of organisms grown in hydrolyzed gelatin and in hydrolyzed casein media. The organisms were cultivated continuously in these media supplemented with 1 per cent blood-cell extract for 528 hours, with a total of nine subcultures in each. The final culture in each was centrifuged, and the bacteria resuspended to T-500. From these suspensions dilutions of 10^{-7}, 10^{-8} and 10^{-9} were prepared. For each medium five mice were injected with each of these dilutions. Each mouse received 0.5 ml. of suspension.

TABLE 7

Titration of virulence in mice of Bactcrium tularense cultured in hydrolyzed gelatin and in hydrolyzed casein media

MEDIUM	NO. OF MICE	DILUTION	DEATH OF ANIMALS IN DAYS														SURVIVALS
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Gelatin	5	10^{-7}	0	0	0	1	2	2									0
	5	10^{-8}	0	0	0	1	2	1	0	1							0
	5	10^{-9}	0	0	0	0	0	1	1	0	0	0	0	0	0	1	2
Casein	5	10^{-7}	0	0	0	0	0	0	4	1							0
	5	10^{-8}	0	0	0	0	0	1	1	1	0	0	0	0	0	0	2
	5	10^{-9}	0	0	0	0	0	1	3	0	0	0	1				0

Table 7 shows that continued subculture in these media effected little decrease in virulence.

SUMMARY

1. Liquid media constructed from either gelatin or casein hydrolysates or from amino acids readily supported growth of *Bacterium tularense* when these media were supplemented with accessory factors. These media are accurately reproducible and contain no protein or other unknown substances of large molecular structure. They are suitable for large scale cultivation of the organisms for chemical analyses.

2. The significant accessory factors are liver-cake extract, blood-cell extract, and biotin concentrate. Pantothenic acid, pimelic acid, nicotinic acid and biotin mixture permitted growth of the organism to a limited degree. However, these could not replace blood-cell extract or liver-cake extract in entirety.

Although all obligate and accessory factors necessary for growth are not yet chemically definable, a close approximation to complete media must have been reached since an inoculum of approximately 1 to 15 bacteria to 5 ml. of medium is sufficient to initiate sustained growth.

3. The accessory growth factors in blood cell extract or in liver-cake extract are thermostable and dialyzable.

4. The indispensability of cystine or cysteine was confirmed. Multiplication of *B. tularense* was never observed in the absence of these amino acids. The present studies demonstrate that homocystine, methionine, or thioglycollic acid could not be substituted for cystine.

5. The sodium chloride tolerance of the organism was found to be high, the optimum amount of sodium chloride being one per cent or higher. Two per cent was not inhibitory.

6. Virulence of the organism was maintained at or near maximal after prolonged continuous cultivation in the liquid media.

7. The production of a soluble toxin by *B. tularense* could not be demonstrated.

8. The production of alkali in liquid media was well demonstrated.

The work is being continued in an effort to identify the components of the blood cell and liver-cake extracts essential for growth.

ACKNOWLEDGMENTS

This research was aided in part by a grant from the Craig Yeiser Memorial Fund.

The authors are indebted to Dr. Y. Subbarow of Lederle Laboratories, Inc. for a generous supply of liver-cake.

One of us (I. G.) wishes to express gratitude to Merck & Co., for furnishing certain amino acids and growth factors.

BIBLIOGRAPHY

BERKMAN, S., AND KOSER, S. A. 1941 Accessory growth factor requirements of the genus *Pasteurella*. J. Bact., **41**, 38–39.

BRIGHAM, G. D., AND RETTGER, L. F. 1935 A systematic study of the *Pasteurella* genus and certain closely related organisms. J. Infectious Diseases, **56**, 225–237.

FRANCIS, E. 1922 Cultivation of *Bacterium tularense* on three additional mediums new to this organism. Hygienic Lab. Bull. No. 130, pp. 83–84.

FRANCIS, E. 1942 Fermentation of sugars by *Bacterium tularense*. J. Bact., **43**, 343–346.

GUNDRY, L. P., AND WARNER, C. G. 1934 Fatal tularemia. Ann. Internal Med., **7**, 837–852.

LUFKIN, N. H., AND EVENSON, A. E. 1936–7 Tularemia diagnosed by routine blood culture. J. Lab. Clin. Med., **22**, 346–350.

MCCOY, G. W., AND CHAPIN, C. W. 1912 Further observations on a plague-like disease of rodents with a preliminary note on the causative agent, *Bacterium tularense*. J. Infectious Diseases, **10**, 61–72.

WILLIAMS, R. ET AL. 1941 Studies on the vitamin content of tissues. 1. Univ. Texas Publ. No. 4137, p. 83. Austin.