flagellar nature of heat labile, flocculating antigens. As yet, no satisfactory explanation can be offered for the nonfunctional nature of the flagella in flagellated microorganisms which give no evidence of motility. Second, some degree of discretion should be exercised in characterizing a culture as being nonmotile. Unless a culture belongs to a well-known type which is known to lack motility, it should be examined carefully and be transferred serially in semisolid medium before it is concluded that motile forms cannot be obtained from it.

Summary. Two apparently nonmotile strains possessed well-developed flagella and flagellar antigens. One of the types (III, XV:e,h) eventually yielded motile elements after serial transfer in semisolid medium. The other *(S. sandiego)* gave no evidence of motility after similar treatment.

15457

Submerged Growth of Tubercle Bacilli from Pathologic Material in Dubos' Medium.

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The recent reports by Dubos[1] describing the remarkably rapid growth of Mycobacteria in a fluid medium containing purified phosphatides or certain synthetic nonionizing surface-active agents suggested the possibility that such media might be useful in the examination of pathologic material in the diagnostic laboratory.

During a period of 3 months 165 specimens were received for routine examination for tubercle bacilli by guinea pig inoculation. These specimens, untreated, were injected into guinea pigs according to the method in routine use. One hundred and forty-three of these specimens were examined by culture as well as guinea pig. For cultural examination, 5-20 ml volumes of the specimen were digested with an equal volume of 3% hydrochloric acid for periods varying from 15 minutes to 2 hours at room temperature, then neutralized to approximately pH 7.0 with 3% sodium hydroxide.[2] The digest was centrifuged and the sediment concentrated to 1/10th to 1/20th the original volume in sterile physiologic saline solution.

Several variations of basic media as described by Dubos[1] were used in this study (Table I). The various substances added to the basic media, together with the number of specimens examined in each are summarized in Table II. The specimens recorded as cultured in each basic medium were cultured in all the variations noted in Table II. Asolectin was first dispersed in a few ml of ether and added to the basic medium before autoclaving. Basic media were adjusted to pH 7.0-7.2 with normal sodium hydroxide and autoclaved at 15 lb. for 15 minutes. Glucose, albumin and Vegex were added to the autoclaved base in the form of sterile solutions. The media were dispensed in ordinary Pyrex test tubes 18-20 mm in diameter, stoppered with nonabsorbent cotton and sterilized in the autoclave. The various media were used in volumes ranging from 1 to 5 ml. Although growth can be obtained in as little as 1 ml of media, larger volumes (3-5 ml) were preferable since many cultures were kept in the incubator for 14-21 days, resulting in considerable reduction in volume by evaporation. Each lot of media was tested for growth-supporting capacity with stock strains of human (H37Va) and bovine tubercle bacilli and an acid-fast saprophyte *(M. phlei)* before

[1] Dubos, R. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 361.

[2] MacNabb, A. L., *Diagnostic Procedures and Reagents*, Am. Pub. Health Assn., 1941, Ed. 1, 281.

TABLE I.

Submerged Growth of Tubercle Bacilli from Pathologic Material: Basic Media.

Base		% in 1000 ml dist. water
1	Asparagine	.5
	Sodium citrate	.6
	Potassium phosphate (Dihydrogen)	.2
	Ammonium chloride	.1
	Magnesium sulphate	.05
	Ferric ammonium citrate	.005
2	Casein hydrolysate*	.2
	Sodium citrate	.15
	Potassium phosphate (Dihydrogen)	.1
	Magnesium sulphate	.06
	Sodium phosphate (Disodium)	.625
	Tween 80†	.05
3	N-Z Amine‡	.2
	Sodium citrate	.15
	Potassium phosphate (Dihydrogen)	.1
	Magnesium sulphate	.06
	Sodium phosphate (Disodium)	.625
	Mannitol	.5
	"Asolectin"§	.05

All minerals CP, not recrystallized.

* Product of Difco Lab., Inc., Detroit, Mich.

† A polyoxyethylene derivative of sorbitan monooleate, product of the Atlas Powder Co., Wilmington, Del.

‡ Product of Sheffield Farms Co., New York City.

§ Soya bean phosphatides, product of Associated Concentrates, Inc., Atlanta, Ga.

being used for culture.

Media were inoculated with 0.1-0.5 ml of the digested, concentrated specimen, these volumes representing from 2 to 10 times the volume of untreated sample as injected into the guinea pigs. In general the smaller volumes of inocula were used for sputa and necessarily, small volumes of specimen, while larger inocula were used for specimens such as urine or gastric aspiration. The inoculated media were incubated at 37°C and examined daily for one week for evidence of growth. Specimens were smeared as soon as visible growth appeared, in any case each was smeared after 2, 7, 14 and 21 days incubation. Smears prepared prior to the 21st day were made from the uncentrifuged culture, the final smears on the 21st day were made from the centrifuged sediment. Ziehl-Neelson or Kinyoun's modification[3] of the Ziehl-Neelson acid-fast stain was used.

Most of the positive specimens examined were obtained from cases in which tuberculosis was suspected clinically, but could not be established by direct microscopic examination of concentrated specimens. A few of the other positive specimens were obtained from cases in which tuberculosis was considered unlikely, the specimen having been submitted for exclusion.

As may be seen in Table II, there was excellent correlation between culture results and guinea pig inoculation, except with those specimens cultured in a medium which did not contain serum albumin. As Dubos observed,[4] the essential role of serum albumin may be merely a detoxifying action on inhibitory substances formed in the medium rather than nutritive; in either case it is evident that a small amount, either human or bovine, is necessary to insure growth. If the results obtained with the 4 specimens which were cultured in this medium are discarded, there remains a total of 29 specimens in the series which were positive either by culture or guinea pig or both. Twenty-six (90%) were positive by both culture and guinea pig. Two additional specimens were positive by culture alone (Table II), hence 28 or 96.6% of the specimens which contained tubercle bacilli were positive by culture. This figure does not differ significantly from the 27 positives (93.6%) obtained in guinea pigs; 26 positive by culture as well as by guinea pig; one, a bladder urine, positive by guinea pig alone. The 2 sputa which were positive by culture and negative by guinea pigs (Table II) were obtained from 2 different patients who previously had sputa or gastric aspirations positive both by culture and by guinea pig. A positive culture with a negative guinea pig on the same specimen can probably be accounted for by the difference in the volumes of inocula used in the culture and guinea pigs, respectively.

The kind and number of specimens with which positive results were obtained are summarized in Table III. The incubation range of from 5 to 27 days, with a mean of 11 days is in sharp contrast to the 8-12-week period

[3] Kinyoun, J. J., *Am. J. Pub. Health*, 1915, **5**, 867.

[4] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

TABLE II.
Submerged Growth of Tubercle Bacilli from Pathologic Material: Specimens Examined in Different Variations of Basic Media.

Medium	No. specimens examined	No. positive by culture	No. positive by guinea pigs
Basic 1 + 2% glycerin, 0.01% "Asolectin" 0.05% " 0.5% glucose, 0.01% " 0.05% "	12	0	4
Basic 1 + above variations, each with 0.2% (human) serum albumin*	45	12	12†‡
Basic 1 + 2% glycerin, 0.05% "Asolectin" 0.5% glucose, 0.05% "Asolectin" each with 0.2% (human) serum albumin	28	7	6†
Basic 2 + 0.5% glucose, 0.2% Vegex,§ 0.2% Bovine Fraction V	36	6	6
Basic 3 + 0.2% Vegex, 0.3% Bovine Fraction V	22	3	3
Total	143	28	31

* By courtesy of the Mass. Dept. of Public Health.
† Two sputa negative in guinea pigs, positive in cultures. In both instances previous specimens on these 2 patients were positive in guinea pigs and culture.
‡ One bladder urine positive in one guinea pig, negative in culture.
§ Product of Vegex Corp., New York City.
‖ Product of Armour Lab., Chicago, Ill.

TABLE III.
Submerged Growth of Tubercle Bacilli from Pathologic Material: Incubation Time of 28 Positive Specimens.

Material cultured	No. specimens	Incubation in days Range	Mean
Sputum	10	5-21	8.3
Gastric aspiration	6	7-27	12.1
Urine*	7	7-20	12.7
Miscellaneous			
Joint fluid	2		
Pus	1		
Autopsy material†	1	6-21	10.6
Epididymis	1		
Total	28	5-27	11.1

* Five bladder, 2 catheterized specimens.
† Tuberculous meningitis. Antemortem and postmortem spinal fluids, pus from arachnoid, periaortic node, uterus and tube all positive. Another case with an antemortem diagnosis (clinical) of tuberculous meningitis was negative by culture. However, *H. capsulatum* was isolated later from an antemortem spinal fluid.

usually required for diagnosis by routine guinea pig inoculation. Twenty-three (82%) of the positive results were obtained in 14 days or less. The 5 specimens requiring incubation periods of more than 14 days (Table IV) were of the kind which usually contain extremely small numbers of tubercle bacilli. The incubation periods observed in this experiment probably represent the maximum since after the seventh day the culture as a rule was not resmeared until the 14th and 21st days. If daily examination by smear, or more frequent examination of the centrifuged sediment were practicable on all specimens, a still further reduction in time might be expected.

TABLE IV.
Submerged Growth of Tubercle Bacilli from Pathologic Material: Positive Specimens Requiring More than 14 Days Incubation.

Material cultured	Incubation in days
Gastric aspiration	18
Bladder urine	20
Sputum*	21
Joint fluid	21
Gastric aspiration	27

* Guinea pigs negative. Previous specimens on same patient positive by guinea pigs and by culture.

The amount of growth obtained with different specimens showed considerable variation, but could not be correlated with differences between the various lots of media recorded in Table II. It seemed that the amount of growth depended more on the nature of the specimen (number of tubercle bacilli in the inoculum?) or perhaps strain differences than on the particular medium used. Some cultures presented massive "rafts" of tubercle bacilli many immersion fields in area, while others required diligent search of the smears before a few small clumps of tubercle bacilli could be demonstrated. In either case the arrangement of cells in clumps connoted reproduction. In all cases, further incubation or transfer of a slowly growing culture to fresh media resulted in a more profuse growth. The morphology of tubercle bacilli grown in these media seemed to be slightly different from that observed on the various coagulated egg media. The individual cells tended to be longer and slightly thicker. Beaded and banded forms, such as are seen in smears made directly from tuberculous lesions, with bipolar staining or large, nonacid-fast granules are common.

Discussion. This preliminary study suggests that with reasonable care in their use, these new synthetic media can be invaluable in the laboratory diagnosis of tuberculosis. The cultural results reported here perhaps are not directly comparable to the results in guinea pigs with regard to sensitivity, since concentrated specimens were compared in culture with the unconcentrated specimens in guinea pigs. Concentrated inocula were used in order to determine whether or not the media would support the growth of tubercle bacilli from pathologic material without attempting a direct comparison of the inocula volume for volume in guinea pigs and cultures.

Digestion for as short a time as 15 minutes at room temperature with 3% hydrochloric acid satisfactorily eliminated concomitant bacteria from specimens such as urine, gastric aspiration and joint fluids which were not grossly contaminated. Shorter periods of digestion were preferable, in view of the possibility of sterilizing a specimen which contained only a few tubercle bacilli. Sulfadiazine, 30 mg %, was added to the media in which certain of the specimens were inoculated without acid digestion. Although a few positive results were obtained in such media, the method seemed of limited value since the Gram negative microorganisms were the most troublesome contaminants in these media. Also, the drug may crystallize on the slide, making staining and examination more difficult.

These media will support the growth of a variety of nonacid-fast microorganisms, such as *B. subtilis,* some strains of *E. coli* and an unidentified aerobic, Gram negative spore-bearing rod. This latter microorganism, a common dust contaminant, not only withstands the usual acid digestion but also produces an abundance of short, narrow, free spores which retain the carbolfuchsin and might conceivably be mistaken for tubercle bacilli. Certain strains of yeast, frequently present in sputum and gastric aspiration also withstand acid digestion and grow profusely in these media. However, the growth of tubercle bacilli has been observed, even in a badly contaminated culture.

As is the case with other preparations, these media also support the growth of acid-fast saprophytes,[1] hence with the present extent of knowledge of the metabolic differences among this group of microorganisms, they cannot be used alone for the specific bacteriologic identification of tubercle bacilli. However, in those instances where specific bacteriologic identification of the acid-fast organism is indicated, the injection of a pure culture of acid-fast organisms, rather than material taken directly from the patient, into guinea

pigs should result in a more rapid diagnosis. It might be noted that the strain of *M. phlei* mentioned earlier produced a yellow-orange pigment in these media, in contrast to the white, nearly colorless growth produced by the human and bovine strains of tubercle bacilli.

Conclusion. The new synthetic media recently described by Dubos for the rapid cultivation of Mycobacteria can be successfully employed to isolate tubercle bacilli from various pathologic material. A combination of rapid culture with guinea pig confirmation where indicated should result in a marked reduction of the time required for the laboratory diagnosis of tuberculosis.

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Significance of Thromboplastic Activity of Antigens Used in Complement-Fixation Tests.

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Most antigens employed in serologic tests, particularly crude preparations, cause nonspecific reactions in varying degree. They are also usually anticomplementary. The underlying cause of these effects has not been understood. In practice, antigens are diluted to avoid the anticomplementary reaction as far as possible and sera are either used in amounts beyond the range where nonspecific reactions occur or the specificity is evaluated on the basis of the reacting dilution. In some instances nonspecific reactions have been avoided by heating sera above their inactivation temperature, to 60° or 65°C.[1,2] In general this is considered poor serologic practice because of the destructive effect of higher temperatures upon antibodies and of the reactivating effect of such heated sera upon the hemolytic activity of complement.

Relationships between the coagulative and complement activity of serum have been previously noted.[3,4] Cephalin, which is highly thromboplastic, inhibits complement to a degree closely parallel to its clotting activity. This thromboplastic activity of cephalin may be markedly enhanced by the addition of normal mammalian sera, even after inactivation at 56°C for 30 minutes.[5] Since many antigens possess thromboplastic qualities it seems likely that their inhibitory action on complement may be due to the enhancing effect of inactivated serum upon these thromboplastic qualities. It has been our experience that reduction in the thromboplastic quality of the tissue extract used in the complement-fixation test for syphilis diminished the number of nonspecific reactions obtained. The purified antigen, cardiolipin, now widely used, does not possess thromboplastic activity.[6]

The interdependence of these phenomena may have practical importance in the serologic study of sera in neoplastic and virus diseases in which tissue extracts are frequently used as antigens. Normal rabbit sera and fresh tissue extracts have been found to fix complement, which led to the conclusion that normal tissue antibodies were present in such animals.[7] Since fresh tissue extracts are highly

[1] Casals, J., and Palacios, R., *Science*, 1941, **93**, 162; *J. Exp. Med.*, 1941, **74**, 409.

[2] Thomas, L., and others, PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 121.

[3] Wadsworth, Augustus, Maltaner, Frank, and Maltaner, Elizabeth, *J. Immunol.*, 1936, **30**, 417.

[4] Wadsworth, Augustus, Maltaner, Frank, and Maltaner, Elizabeth, *J. Immunol.*, 1937, **33**, 297.

[5] Maltaner, Frank, and Maltaner, Elizabeth, *Arch. Biochem.*, 1943, **2**, 37.

[6] Maltaner, Frank. Unpublished data: Report, June 8, 1942.

[7] Kidd, J. G., and Friedewald, W. F., *J. Exp. Med.*, 1942, **76**, 543.