TABLE III.
Effect of Depressant Drugs on Blood Sugar Level of White Mice (Purdue Swiss).

	No. mice used	Dosage mg/kg	Blood sugar mg % (avg)	S.E.
Controls	61	—	173.8	5.9
Chloroform	12	vapor	172.9	4.8
Nembutal	24	100	70.1	3.5
Dial	12	50	208.0	3.6
Chloral hydrate	11	200	195.3	6.6
Na-pentothal	12	75	172.8	4.2

nembutal caused such a marked hypoglycemia is difficult to explain with our present knowledge. First one group of 12 mice was injected with nembutal and when the hypoglycemic action was noted a second group of 12 (at a later date) was injected in a similar fashion. The 2 groups showed identical responses. The effect of nembutal on glycemia level has been noted by others[16] in mice and rats, and even in fasted rats.

Summary. Hemoglobin in 86 and blood sugar levels in 61 nonstarved albino mice of the Swiss strain were determined. The average hemoglobin for the mice is 13.9 g per 100 ml of blood. Blood sugar averaged 173.8 mg per 100 ml of blood in nonfasted mice falling to 108.9 mg in 24 hours. A slight rise occurred between 18 and 24 hours. The effects of 5 central depressants were investigated, the only one of which causing a drop in blood sugar being nembutal.

[16] Long, C. N. H., personal communication.

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Antibacterial Lipids from *Tetrahymena geleii.*

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It is well known that many species of protozoa ingest and destroy myriads of bacteria. Metchnikoff[1] was probably the first to point out the similarity between phagocytosis in higher animals and intracellular digestion by protozoa, although in neither case is the mechanism clearly understood. Opie[2] has demonstrated the presence of proteolytic enzymes in phagocytes but that they are responsible for the killing of bacteria has not been shown. The presence of antibacterial substances within the phagocyte has been postulated but attempts to extract such substances have, in general, yielded preparations of only weak bactericidal or bacteriostatic properties.[3,*] It seems possible that the bactericidal effect of the living phagocyte may be due to conditions within the cell unfavorable to bacteria such as low pH rather than to the presence of a specific antibacterial agent. Although the same may also be true for the protozoa it was decided to test this point by attempting the isolation of sub-

[1] Metchnikoff, E., *L'immunité dans les maladies infectieuses*, Paris, 1901.

[2] Opie, E. L., *J. Exp. Med.*, 1945, **8**, 410.

[3] Rich, A. R., *The Pathogenesis of Tuberculosis*, p. 289, Chas. C. Thomas, Springfield, Ill., 1944.

[*] Nutini and Kreke (*J. Bact.*, 1942, **44**, 661), and Nutini and Lynch (*J. Exp. Med.*, 1946, **84**, 247), have prepared from various animal tissues, especially spleen and brain, substances having antibacterial properties active both *in vitro* and *in vivo*.

TABLE I.
Antibacterial Activity of Lysates of Four Species of Protozoa.

Organism	Antibacterial activity*				
	Staph "H"	*E. coli*	*Klebsiella pneumoniae*	*M. phlei*	*M. tuberculosis* "H37Rv"
Astasia klebseii	0	0	0	0	
Chilomonas paramecium	0	0	0	0	
Euglena gracilis	0	0	0	0	
Tetrahymena geleii W	0	0	0	1/40	1/10
" " H	0	0	0	1/8	1/8
" " GHH	0	0	0	1/8	1/8
" " E	0	0	0	1/10	
" *vorax* V	0	0	0	0	
" " PP	0	0	0	0	

* Activity is expressed in terms of highest dilution inhibiting growth.

TABLE II.
Bacterial Spectrum of Three Active Preparations.

Test organism	Active preparation				
	W7	W8	(Solids determination showed 23.6 mg/ml)	W9-10	(Solids determination showed 28 mg/ml)
Mycobacterium tuberculosis "H37Rv"	1/300*	1/700*	33.7†	1/860*	32.6†
" *phlei*	1/200			1/667	42.
Pneumococcus Type I	1/6800	1/8000	2.95	1/16000	1.13
" " III	1/9500	1/8000	2.95	1/16000	1.13
Streptococcus viridans	1/2500	1/4000	5.9	1/4000	4.5
" *pyogenes* "C-203"	1/1220	1/2000	11.8	1/2000	9.
Staphylococcus "H"	1/54	<1/10		1/154	182.
Escherichia coli	<1/10	<1/10		<1/10	
Salmonella enteritidis	<1/10	<1/10		<1/10	
Shigella dysenteriae Shiga	<1/10	<1/10		<1/10	

* Highest dilution inhibiting growth of test organism.
† Least amount in μg/ml inhibiting growth of test organism.

stances having antibacterial properties.

Bacteria-free cultures of *Astasia klebseii, Chilomonas paramecium, Euglena gracilis,* and *Tetrahymena geleii,* were obtained through the courtesy of Professor George W. Kidder, Amherst, Mass. In preliminary experiments the organisms, grown in a tryptone-acetate or a proteose-peptone broth, were sedimented in the cold, resuspended in 1/10 volume of the clear supernate, and repeatedly frozen and thawed. These lysates were then tested for inhibitory activity against *Staphylococcus aureus, E. coli, Klebsiella pneumoniae* and, in certain cases, 2 strains of mycobacteria, one a virulent human strain and the other a saprophyte.

It will be seen from the data presented in Table I that growth of both *Mycobacterium tuberculosis* and *M. phlei* was inhibited by lysates of *Tetrahymena geleii* but not by lysates made from the other strains. Filtration of these lysates through Seitz filters removed all activity. None of the lysates inhibited growth of the other 3 bacterial cultures tested. The same lysates when tested against a culture of *Photobacterium fischeri* showed no antiluminescent activity.

Since growth of both *M. tuberculosis* and *M. phlei* was inhibited by lysates of *Tetrahymena geleii,* it became of interest to obtain, if possible, preparations of greater purity and potency for more extensive study. For this purpose, Blake bottles containing 200 ml of 1.5% proteose-peptone broth were inoculated with 4 ml of a 24-hour culture of *Tetrahymena geleii* "W" and incubated horizontally for 4-5 days at 25°C. The organisms were then sedimented in a Sharples centrifuge and stored in the frozen state until used. The yield of moist sedimented organisms was approximately 1.3%.

Preliminary experiments showed that or-

ganic solvents (ether, acetone, and methanol) extracted the active material from the lysed organisms and that activity could be re-extracted from the ether solution with aqueous alkaline solutions. Details of extraction will be given in a later section.

Experiments *in vitro* and *in vivo* which have been carried out with various active fractions are presented below.

Activity in vitro. Three solutions which were prepared from sedimented whole organisms in the following manner were tested *in vitro* against a variety of organisms: (1) W7 was a 1% Na_2CO_3 extract of ether soluble material, (2) W8, a water solution of the precipitated sodium salts obtained from an extract of hexane soluble material, and (3) W9-10, a NaOH extract of ether soluble material. A broth dilution test was used. Kirchner's synthetic medium with the addition of Tween 80[4,5] to induce diffuse growth was used for tests with acid-fast organisms. Tests with other organisms were made in a beef heart infusion broth. Both media were well buffered and results were not affected by the alkaline nature of the solutions tested. The cultures used in testing were diluted as follows: acid-fast organisms 1-50, pneumococcus and streptococcus cultures 1-100, and staphylococcus and Gram-negative cultures 10^{-6}. The diluted cultures were dispensed in 13 x 100 mm tubes in 2 ml amounts and the inhibiting agents added with a Kahn pipette in decreasing amounts; usually from 0.1 to 0.01 ml. The incubation temperature was 37°C. Tests with *M. phlei* and *M. tuberculosis* "H37Rv" were read after 3 and 7 days respectively. Other tests were read after 18 hours. The results of these tests are given in Table II.

All preparations showed high activity against the pneumococcus and the streptococcus with a lesser activity against the acid-fast cultures. Two of the preparations showed slight activity against the staphylococcus, but all were inactive against the Gram-negative bacilli.

TABLE III.
Effect of Serum and Blood on Activity *in Vitro*.

Preparation	B.H.B.	B.H.B. 5% serum	B.H.B. 5% blood
W9-10	1.5	>240	>240
W11-12	3.2	90	760
Py7-10-11D	18.5	>250	

Possible inhibition by serum and blood of activity *in vitro* against the Type III pneumococcus was tested. The medium used was a beef heart infusion broth unenriched and with the addition of either 5% horse serum or 5% rabbit defibrinated blood. All of the 3 preparations tested showed greatly reduced activity in the presence of serum or blood. The results given in Table III show the minimum inhibiting concentration in micrograms per ml.

The active preparations described above were all obtained from disrupted protozoa. The culture medium after removal of the protozoa showed no antibacterial activity. However, 2 supernates after Sharples centrifugation were acidified to pH 2.5, extracted with chloroform and the chloroform re-extracted with a small volume of sodium carbonate solution. One such extract (95 ml) obtained from 16 liters showed activity in a dilution of 1-54 against *M. phlei* and another extract (30 ml) obtained from 17½ liters was active in a 1-70 dilution. Since Sharples centrifugation did not remove all the organisms from the culture medium, the slight activity obtained probably was derived from the organisms still remaining in the culture medium.

In the early experiments in which lysates of several species of protozoa were tested for antibacterial activity only those from *Tetrahymena geleii* showed activity. Since preparations of much higher potency were later obtained from *Tetrahymena geleii* by the extraction methods described, the species *Chilomonas paramecium* was subjected to the latter procedure. An extract so prepared proved to be highly active when tested against the Type III pneumococcus. Work with this extract was not continued, however, since it appeared that the active material was similar

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

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

TABLE IV.
Toxicity for Mice of Preparation W9-10.

Dose, mg	Route of inj.	Lived	Died
14	Intraperitoneal	5	0
14	Subcutaneous	5	0
7	Intravenous	0	10
3.5	"	6	4
1.75	"	5	0

in nature to that from *Tetrahymena geleii*.

Toxicity. Preliminary tests on toxicity for mice of preparations W9-10 were made. The results are given in Table IV.

Peritonitis developed in those mice injected intraperitoneally, and cellular infiltration and necrosis of the skin in those injected subcutaneously.

Since there were indications at this point that the substances under investigation were fatty acids, more extensive toxicity studies were deemed unnecessary.

Activity in vivo. Although both serum and blood decreased activity *in vitro* indicating probable inactivity *in vivo*, protection test in mice against *M. tuberculosis* "H37Rv" were carried out with 2 preparations, Py6 and Py7. The minimum inhibiting concentrations of the 2 preparations against *M. phlei* were respectively 25.6 and 73.8 μg/ml. Treatment, which was begun immediately after infection, consisted of the subcutaneous injection of 5 mg per day.

Suspensions of culture made from surface growth on Kirchner's medium were injected intravenously into white mice weighing 17 g. In the first experiment a light infecting dose was used (0.5 ml of a filtrate through Whatman No. 1 filter paper of a 1 mg/ml suspension). Treatment was continued for 26 days. In the second experiment the infecting dose was heavier (0.5 ml of a 1 mg/ml suspension) and treatment was continued for 13 days. Each experiment consisted of 3 groups of 10 mice, one group treated with one of the above preparations, one with streptomycin (5000 units per day given in 3 daily doses), and one untreated group. The mice were sacrificed immediately after treatment was stopped and the extent of lung and spleen lesions noted. In both experiments the mice treated with Py6 and Py7 showed as extensive lesions as the controls while infection in the group treated with streptomycin was retarded.

Inasmuch as all preparations showed high activity *in vitro* against the Type III pneumococcus protection tests in mice against this organism were also performed.

Mice were infected intraperitoneally with 1 ml of a 10^{-6} dilution of a 6-hour blood broth culture. Three partially purified preparations were tested W9-10, Py6 and Py7. Three subcutaneous injections of 5 mg each were given, one immediately after infection and 2 on the following day. No protection was afforded. All treated mice died as quickly as controls.

Chemical Fractionation. In view of the marked *in vitro* activity of these various crude preparations, fractionation experiments were undertaken in an effort to isolate in pure form and identify the active principle.

The following procedure is that eventually devised for the isolation and purification of this acidic material. Acetone was found to be the solvent of choice for the quantitative extraction of the active material from the disrupted organisms. In a typical run, 568 g of the moist organisms were mixed in a Waring Blender with 250 ml of acetone and the brei then centrifuged. This procedure was repeated with 4 more 250 ml portions of acetone. The dried acetone-insoluble material weighed 16.5 g and no longer yielded active material on extraction with various solvents. The combined acetone extracts were concentrated *in vacuo* to remove the acetone and the remaining cloudy aqueous solution was continuously extracted with ether for 24 hours. The aqueous phase, after removal of the ether, showed no inhibiting activity and was discarded. Preliminary experiments had shown that the entire activity could be extracted from the ether solution with 1% sodium hydroxide solution, but with the aim of effecting a fractionation of the acidic materials, preliminary extraction with 1% sodium carbonate solution was carried out.

Ten 25 ml portions of 1% sodium carbonate were used, the extracts combined and reextracted with ether following acidifica-

tion. The ether solution upon evaporation yielded an oily residue weighing 3.1 g. Made up in solution in 1% sodium carbonate at 5 mg per ml this fraction inhibited *M. phlei* at 1 to 68 dilution.

The original ether solution remaining after the extraction with 1% sodium carbonate was extracted with four 25 ml portions of 1% sodium hydroxide solution. These were combined, acidified and reextracted into ether from which was obtained on evaporation a pigmented, oily residue weighing 890 mg. The original sodium hydroxide solution, which therefore contained 8.9 mg per ml, had shown inhibition of *M. phlei* at 1 to 280 dilution.

The ether solution remaining after the 1% sodium hydroxide extraction was washed with water, dried and evaporated. A semi-crystalline residue weighing 1.288 g remained. This fraction showed no inhibiting activity. Crystallization of this material from absolute alcohol yielded 370 mg of glistening platelets of a neutral compound which melted at 309 to 312°. It contained no nitrogen or sulfur and analyzed for a compound of the formula $C_{27}H_{45}OH$, isomeric with cholesterol. The mother liquors left an oily residue which probably consisted of neutral lipids.

The 3.1 g from the sodium carbonate extract were taken up in acetone and the solution centrifuged to remove 108 mg of insoluble material. After treatment with Norite and concentration, the acetone solution deposited several crops of colorless leaflets which were combined and recrystallized from acetone. From 700 mg of crude crystals melting from 49 to 53° there were obtained 150 mg of colorless needles melting at 53-54°. The mother liquors yielded further material of slightly lower melting point. In the original acetone mother liquors there remained 2.4 g of oily acidic material.

The crystalline material of melting point 53-54° contained no sulfur or nitrogen and yielded the following analytical values.

Found: C, 73.85; H,12.60

Calcd. for $C_{13}H_{27}COOH$: C, 73.63; H, 12.36

The neutralization equivalent was somewhat higher than that required for myristic acid indicating that this product may still be a mixture of fatty acids. However, there appears to be little doubt but that this C_{14} fatty acid is the chief component of this fraction.

By the same treatment with acetone, crystalline material was also obtained from the acidic residue obtained by sodium hydroxide extraction of the ether solution. The top fraction of crystals weighed 188 mg and melted at 50-52°; it appeared to be identical in every regard with that previously obtained. It thus appears that no fundamental fractionation was achieved by the sequence of alkaline solutions, and in subsequent preparations the use of sodium carbonate extraction was abandoned.

When the crystalline fatty acid, fraction Py9 (chiefly myristic), was tested for inhibitory activity, it yielded the results shown in Table V. Other purified fatty acids, palmitic, stearic and oleic, were tested for comparison of activity with that of the material from *Tetrahymena geleii*. The results are also shown in Table V. The inspection of these data disclosed a significant qualitative and quantitative difference between the activities of the purified material and the activities of the crude fractions. A comparison of the activities of the saturated fatty acids with those of oleic acid showed the same sort of variations and it became of interest therefore to determine roughly the proportion of saturated to unsaturated fatty acids in the material from *Tetrahymena geleii*. The presence of these latter acids might account for the higher activity of the crude oily fractions. The neutralization equivalent of a pooled sample of crude acids from *Tetrahymena geleii* was determined and found to be 350, indicating an average chain length of 22-24 carbon atoms. A neutral solution of the acid mixture was treated with lead acetate solution to precipitate the fatty acids, whereupon the dried lead salts were separated into ether soluble and ether insoluble portions. The weights of the acids obtained by decomposing the lead salts showed a distribution of 45% saturated fatty acids and 55%

TABLE V.
Bacterial Spectrum. Crude and Purified Preparations Compared with Oleic, Stearic and Palmitic Acids.

Preparation	*M. tuberculosis* "H37Rv"	*M. phlei*	*M. smegmatis*	Pneumococcus Type III	Streptococcus "C-203"	Staphylococcus
W11-12, crude	29.5	70.7	137.4	3.4	19.1	8.2
Py7-10-11D, crude	65.	375.	375.	18.5	80.6	160.
Py14 saturated fatty acids	80.	303.7	405.	11.8	160.	
Py15 unsaturated fatty acids	80.	185.	217.5	8.5	32.5	240.
Py9 crystalline (myristic acid)	127.5	>500.	>500.	5.0	>500.	240.
Oleic acid	46.7	88.	187.	1.4	14.8	15.
Stearic acid	46.3	>500.	>500.	6.5	>1000	
Palmitic acid	33.4	>500.	>500.	6.5	>1000.	

The figures above represent the minimum inhibiting concentration in micrograms per ml. Py14 and Py15 were derived from crude preparation Py7-10-11D. The activity of some of the above preparations was difficult to determine. Py9, stearic and palmitic acids rendered the test medium so turbid that it was difficult to distinguish between turbidity due to the preparation or that caused by growth of the test organism.

unsaturated fatty acids. The inhibitory activity of these fractions, Py14 and Py15 respectively, is given in Table V.

The iodine number of the crude acid fraction, as determined by Hanus' method, was found to be 35. This value corresponds to a mixture of approximately equal amounts of saturated acids and unsaturated acids, assuming an iodine number of about 75 for the latter.

In a recent paper Dubos[6] states that all fatty acids which he has tested exerted a bacteriostatic effect on tubercle bacilli in a protein-free medium, and that unsaturated fatty acids had the most pronounced effect. He further states that this toxicity can be abolished either by esterification or by addition of crystalline serum albumin. Since the medium we have used for testing inhibition of growth of acid-fast organisms by fatty acids contains 0.05% serum albumin, it is probable that a higher degree of inhibition would have been obtained in a medium without serum albumin.

Discussion. Very little information is available concerning the chemical constituents of protozoa and no reports whatever have been found dealing with the lipids of these microorganisms. Our findings are, therefore, of interest in that they augment the general survey of plant and animal lipids so adequately presented by Hilditch in 1940.[7] While no thorough and systematic fractionation of the lipid material from *Tetrahymena geleii* was made, this fraction of the cellular components could be characterized as follows:

1. The lipid fraction represents from 15 to 20% of the cellular solids.

2. Of this fraction nearly 75% is free fatty acids with only 15% as neutral fat and the remainder a neutral steroid-like compound.

3. The fatty acid fraction represents a complex mixture of saturated and unsaturated acids from which myristic acid has been crystallized. Titration values indicate a preponderance of longer chain acids with an average length of 22 carbon atoms.

These characteristics are in general agreement with observations on other microorganisms; the lipids of the tubercle bacillus, according to Anderson,[8] "consist chiefly of a complex mixture of *free* fatty acids, with relatively small amounts of neutral fat;" and, quoting Hilditch,[7] "the fats of the simplest and most primitive organisms are usually made up from a very complex mixture of fatty acids. Palmitic, myristic and stearic acid are often present in fats of aquatic origin, as also may be unsaturated C_{14} and even C_{24} acids."

The observations reported in this paper concerning the bacteriostatic activity of these fatty acids are in agreement with other simi-

[6] Dubos, R. J., *J. Exp. Med.*, 1947, **85**, 9.

[7] Hilditch, T. P., *The Chemical Constitution of Natural Fats*, Chapman and Hall, Ltd., London, 1940.

[8] Anderson, R. J., *Chem. Rev.*, 1941, **29**, 225.

lar observations recorded in the literature.[9-12] The failure to obtain protection *in vivo* against infections by organisms which are very susceptible *in vitro* to the action of these agents has also been the common finding.[13]

The bactericidal activity of the fatty acids and, since they are usually tested in alkaline or neutral media, their salts, has been recognized as primarily a function of their surface tension lowering capacity[14] with no great specificity attending their molecular structure.[15,16] In this regard they behave similarly to the synthetic detergents[17] and to some of the more recently discovered antibiotics such as subtilin, tyrocidin and gramicidin.[18,19] The explanation of their almost negligible activity *in vivo* appears to lie in their ready absorption by proteins[5,6,20] or through complex formation with phospholipids.[21]

Summary. 1. A mixture of fatty acids which inhibited the growth of mycobacteria was obtained from lysed cultures of *Tetrahymena geleii*. 2. This material could be extracted with organic solvents from the disrupted organisms and purified by reextraction into dilute alkaline solution. 3. The fatty acids in the mixture had an average chain length of 22-24 carbon atoms and represented an approximately 50/50 mixture of saturated and unsaturated acids. Myristic acid was isolated in crystalline form. 4. Partially purified preparations were active *in vitro* against pneumococci, streptococci, mycobacteria and staphylococci, but not against Gram-negative bacilli. Activity was greatly reduced in the presence of blood or serum. 5. No activity *in vivo* against infection of mice with mycobacteria or pneumococci could be demonstrated.

9 Eggerth, A. H., *J. Exp. Med.*, 1931, **53**, 27.

10 Hettche, H. O., and Weber, B., *Arch. Hyg. Bakt.*, 1940, **123**, 69.

11 Drea, W. F., *J. Bact.*, 1944, **48**, 547; 1945, **51**, 507.

12 Franke, W., and Schillinger, A., *Bioch. Z.*, 1944, **316**, 311.

13 Hart, P. D'Arcy, *Brit. Med. J.*, 1946, 849.

14 Stanley, W. M., and Adams, R., *J. Am. Chem. Soc.*, 1932, **54**, 1548.

15 Valko, E. I., *Ann. N. Y. Acad. Sci.*, 1946, **46**, 347.

16 Hotchkiss, R. D., *Ann. N. Y. Acad. Sci.*, 1946, **46**, 530.

17 Shelton, R. S., Van Campen, M. G., Tilford, C. H., Long, H. C., Nisonger, L., Bandelin, F. J., and Rubenkoenig, H. L., *J. Am. Chem. Soc.*, 1946, **68**, 753.

18 Anderson, H. H., Villela, G. G., Hansen, E. L., and Reed, R. K., *Sci.*, 1946, **103**, 419.

19 Hotchkiss, R. D., *Adv. Enzym.*, 1944, **4**, 193.

20 Bergström, S., Theorell, H., and Davide, H., *Nature*, 1946, **157**, 306.

21 Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **74**, 621.