TABLE I.

Strains of Mycobacteria	Long's medium (2% glycerine or 0.5% glucose). +									
Saprophytic	0	-0.05%	Soya	bean	phosphatide	+	0.02-0.2	%	Tween	60
Avian	0	-0.05%	"	"	"	+	0.02-0.1	%	"	"
Bovine and Human	0.01-0.05%		"	"	"	+	0	-0.002%	"	"

the inoculum on the surface of the fluid as is the usual practice with these organisms. The cultures grow readily throughout the fluid and can be transferred repeatedly into the same media. Addition of 0.1 cc of culture to 10 cc of new medium is sufficient to secure growth of the saprophytic and avian strains within 24 hr, and of the bovine and human strains within 72 hr; growth is slower with smaller inocula.

We have also established that the addition of purified serum albumin (0.1% or less) to these liquid media further enhances the growth of tubercle bacilli and permits in particular more rapid multiplication of small inocula.

The cultures obtained under these conditions (with and without serum albumin) are typical in morphology and staining characteristics. When returned to the classical Long's medium (in the absence of the lipid fractions), they manifest again their typical mode of growth, developing slowly, in the form of heaped masses, on the surface of the medium.

Summary. Addition to Long's synthetic medium of small amounts of phosphatides and of long chain fatty acids esters of polyhydric alcohols permits submerged and rapid growth of tubercle bacilli; the different groups of mycobacteria appear to exhibit differential optima with reference to these two types of substances.

14956

The Microtechnical Demonstration of Sites of Lipase Activity.*

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It is known that lipase is not destroyed by acetone dehydration; in fact, the enzyme is usually prepared from acetone-dried tissues. Nor is it too sensitive to heat; in preliminary experiments no appreciable decrease in activity was observed when acetone-dried pancreas powder was kept at 62°C for 2 hr. On the basis of these facts it appeared feasible to demonstrate lipase in paraffin sections by following the principles of the microtechnical demonstration of phosphatases.[1-3] These include the incubation of the slides with an ester, known to be split by the enzyme, in the presence of a salt the metal ions of which form an insoluble salt with the liberated acid and in this way trap the ions of the acid *in situ*. However, when it was attempted to put these principles into practice, great difficulties were encountered. The substrate must meet three requirements which, for a long time, seemed to be mutually exclusive. First, it must be hydrolyzed by lipase; second, it must be water-soluble since lipase is greatly inhibited even by traces of non-aqueous solvents such as alcohols or ketones;[4,5] third, it must form insoluble salts with some metal, non-toxic to the enzyme, such as Ca or Mg. Monocarboxylic acids which have water-soluble esters (up to

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1 Takamatsu, H., *Tr. Soc. Path. Jap.*, 1939, **29**, 492.

2 Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 23.

3 Gomori, G., *Arch. Path.*, 1941, **32**, 189.

4 Murray, D. R. P., *Biochem. J.*, 1929, **23**, 292.

5 Glick, D., and King, C. G., *J. Biol. Chem.*, 1931, **94**, 497.

C_4) do not have insoluble salts. The esters of those forming insoluble salts (from C_{12} upward) are insoluble in water. Of the dicarboxylic acids, the water-soluble esters of oxalic acid, the only acid having a highly insoluble Ca salt, are not attacked by lipase.[6] The salts of the higher dicarboxylic acids which have water-soluble, readily hydrolyzed half-esters (glutaric, adipic, sebacic) are not sufficiently insoluble for the purpose of microtechnical demonstration. A number of attempts with dispersions of water-insoluble esters were uniformly unsuccessful. At this point it was learned that a number of water-soluble long-chain fatty acid esters of polymer glycols and hexitols have recently become available commercially. Samples of several of these compounds were obtained all of which were found to be readily hydrolyzed by a water-glycerol extract of acetone-dehydrated pancreas. A microtechnical method for the demonstration of lipase has been developed by using Tween 40 or Tween 60, palmitic and stearic esters, respectively, of hexitans in which most of the hydroxyl groups are etherified. The Tweens,[7] manufactured by the Atlas Powder Co., Wilmington, Del., are amber colored, semisolid, unctuous substances which give crystal clear aqueous solutions.

The Tweens were found to be hydrolyzed by pancreatic lipase at a rate about half that of olive oil as determined by the method of Sure, Kik and Buchanan.[8]

The following technic was found to yield uniformly good results:

1. Fix thin slices of fresh tissues in ice cold absolute acetone for 12 to 24 hr. Dehydrate at room temperature in 2 more changes of absolute acetone, 6 to 12 hr each. Embed through 2 changes of benzene, 30 min each, in paraffin. Neither 60°C, nor 2 hr at this temperature should be exceeded.

2. Cut sections around 5 micra. Pass slides through xylene and 2 alcohols to distilled water. Dip slides between the first and second alcohol into a dilute (0.5%) solution of collodion in alcohol-ether.

3. Incubate slides for 6 to 12 hr at 37°C in the following solution:

2% solution of Tween 40 or 60	5 cc
M/10 maleate buffer pH ± 7.4,	
30% glycerol	20 " each
2% solution of $CaCl_2$	5 "

This solution may be slightly hazy at 37°C. During incubation, the sites of lipase activity will become opaque owing to the precipitation of Ca palmitate or stearate.

4. Rinse slides in distilled water.

5. Transfer to a 2% solution of lead nitrate for 10 min. Ca palmitate or stearate are transformed into the corresponding Pb salts.

6. Rinse slides in many changes of distilled water.

7. Transfer to a dilute solution of yellow ammonium sulfide (about 10 drops to a Coplin jar filled with distilled water) for 2 min. Lead palmitate and stearate will be transformed into dark brown lead sulfide.

8. Wash under the tap. Counterstain with hematoxylin. Dehydrate and mount. It is advisable to use ligroin or dichloroethylene for clearing, and clarite or some similar resin dissolved in the same solvents for mounting, since the stain will gradually fade in media containing xylene.

So far, mainly normal dog and rat organs have been examined by this method. Lipase could be localized in many organs such as liver, pancreas, lung, kidney, testicle, epididymis, adrenal, adipose tissue, stomach and small intestine. In the liver, the largest amount of lipase is found in the central portion of the lobules (Fig. 1). In the pancreas, the site

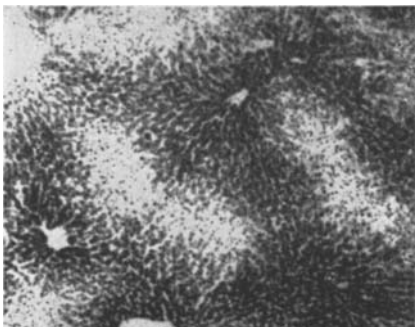

FIG. 1.

[6] Westenbrink, H. G. K., and Romijn, H. M., *Arch. néerland. de phys.*, 1930, 15, 529.

[7] Atlas Spans and Atlas Tweens. Atlas Powder Co., 1943.

[8] Sure, B., Kik, M. C., and Buchanan, K. S., *J. Biol. Chem.*, 1935, **108**, 27.

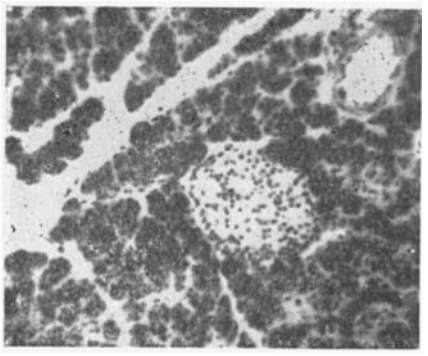

FIG. 2.

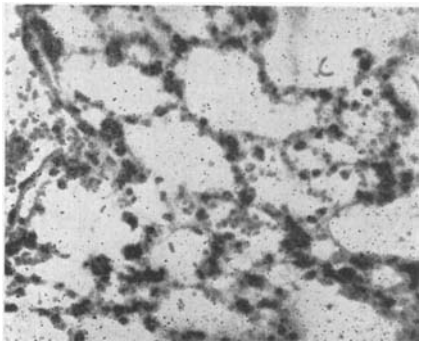

FIG. 3.

of the enzyme coincides with that of the zymogen granules, while the islets of Langerhans are entirely negative (Fig. 2). In the lung, the bronchial epithelium and the septal cells are stained selectively (Fig. 3). No lipase was demonstrated in the spleen, lymph nodes, brain and muscle.

The extent and intensity of the histochemical reaction is in good agreement with the results of chemical determinations of lipase activity in the same organs. Dog liver, rat kidney and rat pancreas which show a very intense and extensive histochemical picture were found to liberate 1.6 to 2.6 mM of butyric acid in 2 hr per g of tissue from methyl butyrate buffered at pH 7.6. Rat lung and dog kidney which show much less activity in the slide were found to liberate 0.45 to 0.84 mM of acid under the same conditions. Rat testicle and guinea pig kidney which show even less activity microscopically, liberated 0.15 to 0.35 mM of acid. Finally, brain, muscle and lymphatic tissue, negative histologically, showed only traces of lipase activity in the test tube (less than 0.05 mM of acid).

The reaction is completely prevented by treating the slide before incubation with Lugol's iodine solution for one minute, or with 5% phenol or with boiling water for 10 min.

When the effect of bile salts on the lipase picture of various tissues was studied, it was found that the addition of 0.2% Na taurocholate to the incubating mixture lead to a considerable intensification of the reaction in the pancreas, while in all other organs the enzyme was greatly inhibited. This is in good agreement with known differences in the bile salt activation of pancreatic and other lipases.[9]

Summary. Lipase can be demonstrated microtechnically in paraffin sections of acetone-fixed tissues. The slides are incubated with a water-soluble ester of palmitic or stearic acid in the presence of $CaCl_2$. At the sites of lipase activity, the insoluble Ca palmitate or stearate precipitates can be visualized by transformation into the corresponding lead salts and subsequent blackening with H_2S.

[9] Willstaetter, R., and Mennen, F., *Z. physiol. Chem.*, 1924, **138**, 216.

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