Chemistry of Acid-Fastness.* (20588)

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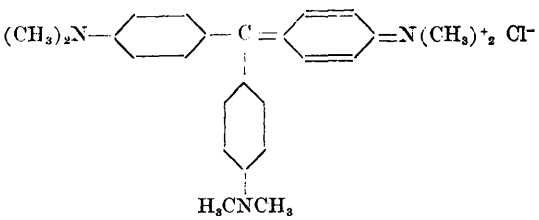
Recent work from this laboratory(1) has shown that the acid-fastness seen in mycobacteria resided in two different properties of the cells. First, there was an acid-fastness dependent upon cell structure, and hence shown by intact but not by crushed tubercle bacilli. Secondly, in the same cells there was an acid-fastness not altered by structural changes but rather dependent upon chemical composition. This second type of acid-fastness was lost only when compounds of the mycolic acid type were removed from the cells. Further, purified mycolic acid showed this same type of acid-fastness to the same degree. Lepra bacilli and sperm showed only the second type of reaction corresponding to the reactions respectively of leprosinic acid and the similar lipid recently found in human sperm(2).

The next logical step seemed to be a study of the reaction between mycolic acid and a basic dye. Contrary to previous beliefs(3) a stoichiometric relationship was found to exist between acid and dye. In addition the complex formed between mycolic acid and the dye was found to have specific and particular properties that set it off from combinations of the same dye with other organic acids.

Materials. Several samples of mycolic acid and leprosinic acids as well as the model acids tested were obtained from Dr. E. Mylon of this department. Highly purified leprosinic acid and the methyl, acetyl, and methyl-acetyl derivatives thereof were generously provided by Dr. C. A. Hargreaves, Dept. of Chemistry.

Stain-Commission certified crystal violet (C.I. 681, lot No. NC 32) was the dye chosen for the study. Although all basic dyes give the acid-fast reaction(4), crystal violet had several advantages. It was the only dye, brilliant in the visible range, which was completely removed from non-acid-fast materials by the standard acid-alcohols. Most other dyes left some residual stain in all non-acid-fast tissues, and the fuchsins left a large amount. This absence of non-specific staining offered the hope that there might also be fewer complicating chemical reactions encountered. Further the dye had a symmetrically resonating structure:



which it was thought might simplify the interpretations of the results.

Results. A. *Determination of dye-acid equilibria.* The method, adapted from Glasstone(5), was based on the fact that while the dye was soluble both in water and chloroform, the mycolic and leprosinic acids were soluble only in the latter solution.

Approximately 10 mg of dye was accurately weighed and dissolved in 500 ml of water. 100 ml of this solution was mixed with 25 ml of chloroform. In a second flask, 150 ml of the aqueous dye was added to 25 ml of chloroform containing about 10 mg of the acid in question. The movement of the dye from water to chloroform was determined colorimetrically for both systems. Equilibrium was reached for the partition of dye between water and chloroform in 6 days at room temperature (27 ± 2°C) and in 10 days for the acid-dye systems.

In the calculations it was assumed that the distribution of free dye in the two solutions was constant over the range of dye concentration studied, and that the amount of free dye in the chloroform layer was dependent on the concentration of dye in the aqueous layer

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TABLE I. Values at Equilibria for the Reaction between Acid-Fast Lipids and Crystal Violet.

Acid	mg acid/ mg dye*	g acid/ mole of dye	Molecular wt acid and source
Mycolic	3.20	1300 ± 100	
	3.16	1290 ± 100	1284 (6)
Leprosinic	2.97	1210 ± 100	{1250 / 1350} (7)

* Dye wt corrected to a purity of 91% (by titration) as given by the certification analysis. Dye molecular wt 408.

but not on the amount of dye combined with the acid. If these assumptions were true, since the amounts of dye in the aqueous layers at the beginning and the end of the experiment were known, the amount of dye in the chloroform layer could be calculated. That in excess of the dye in equilibrium with the water would be combined with the mycolic or leprosinic acid. The results of the experiment are given in Table I. It is seen that the amount of fatty acid equivalent to one mole of dye was approximately equal to the molecular weight of the acid. This ratio was *independent* of the amount of excess dye present. Hence, within the limits of the technic the reaction may be considered to have gone to completion. Most striking perhaps was the fact that the 1:1 ratio held between dye and leprosinic acid although that acid is known to possess 2 carboxyl groups. This suggests that the 2 acid radicals are not equivalent. The difference may lie in the other reacting groups near the active carboxyl (see below). In any case, the reaction appeared different enough to merit further investigation of the complex which was formed.

B. *Characterization of combination between crystal violet and acid-fast lipids.* The complex formed between dye and mycolic or leprosinic acid was soluble as were the acids themselves although the dye alone was not. When one of these acids was added to xylene layered over the dry dye, the complex was rapidly formed as evidenced by the appearance of intense color in the solution. Excess dye was then removed by filtration. Xylene was removed by evaporation. The material was redissolved in chloroform, the common solvent for the spectral determinations reported hereafter. The visible and ultra-violet spectra of the complexes, the pure acids, and the dye were determined with a Cary recording spectrometer. The results are shown in Fig. 1. The complexes showed the same absorption peak at 5900 R. as did the pure dye. In addition there was even more absorption in a band centered about 3500 Å which did not correspond to any similar band in the spectrum of the pure dye. Neither did mycolic or leprosinic acid absorb at or near this point. The specificity of this new adsorption band was emphasized in the study of reactions of other fatty acids. Cerotic acid, chaulmoogric acid, hexacosanoic acid, 10-methyl-hexacosanoic acid, 10-methyl docosanoic acid, cetyl benzylacetic acid, methyl behenic acid, α methyl-arachidic acid, α methyl tetracosanoic acid, α methyl stearic acid, α methyl cerotic acid, and 10 methyl tetracosanoic acid were all examined and found to be non-acid-fast. Decylmalonic acid, cetylmalonic acid, and hydnocaprylmalonic acid appeared acid-fast and formed a xylene-soluble complex with crystal violet. However, these complexes showed the same absorption curve in the U-V range as did the pure dye, with no evidence of absorption at 3500 Å.

C. *Nature of groups involved in the dye-leprosinic acid complex.* It was recently reported(8) that the carboxyl group was necessary for the reaction between dye and mycolic acid but that the hydroxyl group on the acid could be blocked without affecting acid-fastness. Since the only test of the reactivity had been staining itself, the problem was reexamined to determine whether blocking of either carboxyl or hydroxyl groups would affect the solubility and/or absorption of the

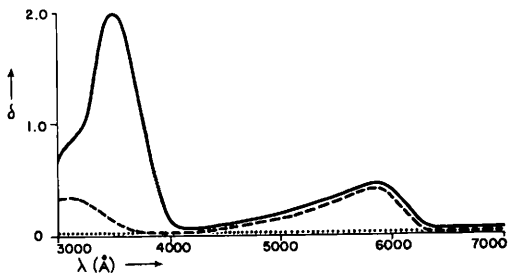

FIG. 1. Absorption spectra (in chloroform). ——— Crystal violet and mycolic acid, crystal violet and leptosinic acid. – – – – – Crystal violet, crystal violet-decylmalonic acid, crystal violet-hexacosanoic acid. ..... Mycolic acid, leprosinic acid.

product. The purified leprosinic acid gave the reactions described above. When the 2 carboxyl groups were blocked by methylation, the substance no longer formed a xylene-soluble complex with the dye, nor did a mixture of dye and ester in chloroform show absorption at 3500 Å. More surprising, exactly the same thing occurred with the compound when one hydroxyl group had been blocked by acetylation although both carboxyl groups were free. Therefore, both the acid and the hydroxyl groups appeared necessary for the *definitive* dye-leprosinic acid reaction.

Discussion. The constant stoichiometric combination of dye and acid-fast lipid, the altered solubility of the complex, and the appearance of the completely new absorption band all speak not only for the uniqueness of the reaction but for the stability of the compound which is formed. It is this stability which is described by the term "acid-fastness" as it refers to the relative difficulty with which the complex is separated by acid hydrolysis. Especially if the new absorption peak is interpreted as signifying a new resonating system in the complex, the union between mycolic acid and dye would appear far different from simple ionic relation ordinarily postulated for basic dye and acid substrate. Whether or not the specificity of the reaction lies wholly in the juxtaposition of carboxyl and hydroxyl groups, both appear necessary for the reaction and so must be considered in the formulation of any theoretical resonating structure. The present information appears insufficient however to describe the complete structure of the dye-mycolic acid complex.

Summary and conclusions. A study was made of the reaction between crystal violet and lipids of the mycolic acid type, considering this to be a prototype of the more general reaction between basic dye and acid-fast lipid. The reaction went to completion in chloroform and at the end 1 mole of dye had combined with 1 mole of mycolic or leprosinic acid. The complex so formed was soluble in xylene as well as chloroform although the dye had been soluble only in the latter solvent. The complex exhibited an intense absorption band in the near ultra violet (3500 Å) which was not present with either the pure dye or pure acids. Other acids were tested and while they might react with the dye, the specific adsorption peak was not produced. If either the carboxyl groups or the hydroxyl group of leprosinic acid were blocked the characteristic reaction did not occur.

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