5. The specific rotation of both the low and high melting point gluconononitriles in pyridine was practically the same, +6.03° and +6.27°, and constant.

6. When gluconononitrile, m. p. 120.5°, was recrystallized four times from acetic acid, gluconononitrile of m. p. 145° was obtained. The latter in water solution had constant rotation.

7. When gluconononitrile, m. p. 145°, was recrystallized four times from absolute alcohol, gluconononitrile of m. p. 120.5° resulted. The latter in water solution showed change in optical rotation. In the authors' knowledge no such unusual reversal of rotation has been observed before in mutarotating sugars.

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[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, COLLEGE OF ST. TERESA]

Chemical Studies of the Dissociants of the H-37 Human Tubercle Bacillus[1]

BY GUSTAV J. MARTIN

In recent years a large number of papers have appeared describing polymorphism and morphological instability in the etiological agent of tuberculosis. The dissociation of *Mycobacterium tuberculosis* into virulent S and avirulent R types by Petroff[2] changed the aspect of chemical investigation of that organism. It was deemed possible that the predominance of one form in any culture might entirely change the outcome of an experiment. Cooper[3] undertook the investigation of the R and S forms of the bacillus of Calmette-Guerin. He encountered insurmountable technical difficulties, but by treating each type in exactly the same manner he was able to get comparative if not quantitative values. The R or avirulent type produced more lipid than the S or virulent type. Differences were found also in the culture medium after growth, the S type elaborating a greater quantity of carbohydrates and soluble substances in general than the R type.

The objective of this preliminary work on the chemistry of the variants of the human (H-37) tubercle bacillus was to subject both to exactly the same treatment and attempt an estimation of quantitative differences. It is our working hypothesis that virulence is a quantitative factor and not a qualitative factor, as is commonly held. Virulence is probably dependent on the presence in the virulent variant of a larger percentage of a certain component. The two variants used, designated as Rv (virulent) and Ra (avirulent), were isolated several years ago and have maintained their biological and morphological characteristics throughout this period. Transplants of stabilized cultures of the two variants were made on the synthetic media used successfully by Petroff. Asparagin is the sole source of nitrogen in this medium. Its composition is as follows

KH_2PO_4, g.	5.0
$MgSO_4$ anhyd., g.	0.6
Mg citrate, g.	2.5
Asparagin, g.	5.0
Glycerol, cc.	20

Dilute to 1 liter with distilled water.

A luxuriant growth appeared in six weeks at 37.5°. The bacteria were then separated from the liquid medium by filtration first through paper and then through Berkefeld candles. After thorough washing, the bacterial residues were dried *in vacuo* over sulfuric acid to constant weight. The bacteria were then placed in thimbles and the Soxhlet extractions started. The entire procedure from initial separation of the bacteria from the media, to the final extractions, was carried out under carbon dioxide. Freshly distilled organic solvents were used throughout. Specially designed covers for the thimbles were added to the ordinary Soxhlet extraction set-up to prevent spattering of bacilli over the sides of the thimble. The extractions were continuous for eight hours per day, and for six days a week. The extraction with each solvent was continued until no further detectable quantities of lipoid appeared. As indicated in Table I, the initial extractions were made in Soxhlet extractors using ether as the solvent. This continued over a six-week period. A second Soxhlet extraction was made with acetone and was continued for a second six-week period. Next, the bacteria which previously had been extracted with both ether and acetone were treated with boiling chloroform for a period of three weeks (eight hours daily). Finally, a reëxtraction with the solvents acetone and chloroform was made, using the liquids at the boiling point. This reëxtraction was motivated by the work of Hecht,[4] who has presented evidence indicating the presence of sterols in the tubercle bacillus.

(1) This study was made possible by a grant from the Committee on Scientific Research of the American Medical Association.

Bacteria grown by William Steenken, Jr., were contributed by the Trudeau Foundation, Trudeau, N. Y.

(2) S. A. Petroff, *Proc. Soc. Exptl. Biol. Med.*, **24**, 632 (1927).

(3) Frank B. Cooper, *J. Biol. Chem.*, **88**, 485 (1930).

(4) Eugen Hecht, *Z. physiol. Chem.*, **231**, 29, 279 (1935).

TABLE I

LIPOIDS OF THE Ra AND Rv VARIANTS OF THE HUMAN H-37 TUBERCLE BACILLUS

Lipoid	19.0 g. of avirulent or Ra variant Amount in g.		% of bacilli	% total lipoid	16.6 g. of virulent or Rv variant Amount in g.		% of bacilli	% total lipoid
Extractable by ether in Soxhlet	1.720	C 0.953 N .0045	9.05	54.5	3.130	C 1.400 N 0.00	18.9	77.2
Extracted lipoid was			Acid fast				Acid fast	
Extractable by acetone in Soxhlet	0.550	C .120 N .00	2.89	17.5	0.630	C .100 N .00	3.8	15.5
Extracted lipoid was			Acid fast				Acid fast	
Extractable by boiling with chloroform	.340	C .158 N .0	1.79	10.8	.075	C .039 N .002	0.45	1.84
Extracted lipoid was			Acid fast				Non-acid fast	
Reëxtraction by boiling acetone	.195		1.02	6.2	.135		0.81	3.32
Extracted lipoid was			Acid fast				Non-acid fast	
Reëxtraction by boiling chloroform	.350		1.84	11.1	.095		0.57	2.34
Extracted lipoid was			Acid fast				Non-acid fast	
Total extractable	3.155		16.6	100.0	4.065		24.5	100.0
% in dry bacteria	16.6				24.5			

Each of the five extracts was evaporated to dryness in partial vacuum under carbon dioxide and each was tested by stained smears to determine the acid fastness of the lipoid and the number of bacteria present. The repeated filtrations of the extracts through multiple thicknesses of fine texture filter paper had removed the bodies of the bacteria, almost quantitatively. The number of acid-fast bacilli was so insignificant that they could not affect materially the results, and since their frequency was approximately the same in both the Ra and the Rv lipoid fractions the results of the analysis are comparable. To eliminate the cells by the use of ultrafilters would introduce a greater source of error in the analysis, because of absorption on the filter, transference difficulties, etc. It was therefore decided that the error introduced by the presence of a few bacterial cells would be of lesser magnitude than that created by the use of ultrafilters.

A sterol test, the Liebermann–Burchard, was made on each lipoid fraction from both the Ra and Rv variants. Each lipoid fraction was analyzed for carbon and nitrogen content, using the gasometric, manometric procedure of Van Slyke.[5] As the chief nitrogen-containing lipoids present in the tubercle bacillus are the phosphatides, a nitrogen determination could be used as an indication of the extractability of the phosphatides in the two cases. Carbon determinations were used mainly as an index of purity as they would have little differential significance in allocating a particular lipoid fraction to any certain extract.

The fluid media were analyzed for nitrogenous constituents: ammonia nitrogen, amide nitrogen, combined ammonia-amide-amino nitrogen and total nitrogen determinations were made. These values permitted direct or indirect estimation of ammonia nitrogen, amide nitrogen, amino nitrogen and protein nitrogen. Analysis for carbon and nitrogen by the manometric method was made on the carbohydrate isolated from the media, and on the purified protein derivative (P. P. D.) isolated therefrom. No determination of inorganic constituents was made, as the amount of ash was the same in both media. It is to be emphasized that the determinations made are not exact, as technical difficulties in handling the bacteria and media preclude all possibility of exact quantitative measurements until improvements in technical procedures are made. However, certain gross differences were readily detected.

(5) J. P. Peters and D. D. Van Slyke, "Quantitative Clinical Chemistry," Vol. II, pp. 385 and 433.

Results

The Lipoids.—There are great differences in the total amount of lipoid extractable from the two variants. The very appearance of the Rv variant colonies would suggest this. By the use of simple organic solvents, 24.5% of the Rv and 16.6% of the Ra organism is extractable. It is to be noted that there is a further small amount of lipoid extractable, only after treatment with hydrolyzing agents, but this would not change the values by 0.5%. Nearly 8% more of the Rv (virulent) organism is made up of lipoid materials. The ease with which the lipoid is extractable from the Rv variant as contrasted with the difficulties encountered in extracting from the Ra furnishes a basis for speculation. In the case of the Rv organism, 18.9% of the dry weight and 77.2% of the total amount of lipoid is extractable by cold ether, as contrasted to only 9.05% of the dry weight and 54.5% of the total amount of lipoid of the Ra organism. A large amount of the nitrogen-containing lipoid, phosphatide, appeared in the first extract from the Ra organism but not in the first extract from the Rv organism. Cal-

culation of the amount of phosphatide in the extract, using Anderson's value of 0.40% for the nitrogen content of purified phosphatide, would give a value of 1.1 g. of phosphatide extracted from the Ra organism by cold ether. The nitrogen-containing lipoids present in the Rv organism were not removed by extraction with cold ether; they appeared in the boiling chloroform extract but in a decidedly smaller quantity, equivalent to only 0.5 g. It is to be noted from Table I that the waxes which are responsible for acid-fastness in the tubercle bacillus were removed from the Rv organism before the third extraction, which was made with chloroform. Extractions with cold ether and cold acetone removed all the acid-fast material as shown by staining reactions. For the Ra organism, we find that all of the extracts contained some of the waxes responsible for acid-fastness in the organisms. The ease with which the lipoids of the Rv variant can be extracted, as contrasted with the more difficult extraction of the lipoids from the Ra organism, may bear some relation to virulence.

Concerning the Liebermann-Burchard tests for sterols, the reactions were decidedly positive on the second chloroform extracts. (These extracts were obtained by the use of boiling chloroform on the organism previously extracted with ether, acetone, boiling chloroform and boiling acetone.) At the suggestion of Dr. Anderson, the corks used in connecting flasks and Soxhlet extractors were subjected to prolonged extraction with chloroform. This chloroform extract was found to give a positive sterol test. Therefore, we assume that sterols in our extracts were a result of contamination. All extracts of the tubercle bacilli probably contained sterols, but with the first two extracts the sterol color test was obscured by the presence of relatively large amounts of neutral fat. We point this out as a possible explanation of the failure of Hecht[4] to confirm Anderson, *et al.*,[6] who found no sterols in tubercle bacilli.

Media Analysis

The yield of dry bacteria from equivalent amounts of the medium would indicate that the Ra form utilized the available carbon and nitrogen for the synthesis of bacterial cells more readily than did the Rv form. Differences are noted in the amount of nitrogen utilized by the two variants; the Rv form seeming to use more nitrogen in its metabolism than does the Ra dissociant. As shown by Table II, the utilization of amide nitrogen by the Rv organism greatly exceeded that of the Ra. The nitrogen utilized per gram of dry bacteria was, in the case of the Rv variant, 0.103 g.; while in the case of the Ra variant the value was but 0.054 g. This would indicate a marked difference in the chemical composition of the proteins present in the two organisms, provided all the nitrogen utilized goes toward the formation of bacillary protein.

TABLE II

MEDIA ANALYSIS

Values represent grams of constituents recovered from media.

Value	Avirulent or Ra variant	Virulent or Rv variant
Yield of dry bacteria from five liters of media	19.0	16.6
Total nitrogen content of media before growth	5.3	5.3
Total nitrogen content of media after growth	4.28	3.59
Nitrogen incorporated in the bacterial cells (by diff.)	1.02	1.71
Amino nitrogen in media before growth	2.65	2.65
Amino nitrogen in media after growth	1.58	1.47
Amino nitrogen converted	1.07	1.18
Amide nitrogen in media before growth	2.65	2.65
Amide nitrogen in media after growth	1.03	0.76
Amide nitrogen converted	1.62	1.89
Ammonia nitrogen in media after growth	1.10	0.96
Amino, amide and ammonia nitrogen in media after growth	3.71	3.18
Protein nitrogen in media after growth	0.57	0.41
Protein in media after growth calcd. from protein nitrogen	3.56	2.54
Ash in media after growth	28.0	28.5

Discussion

As previously stated, Cooper[3] obtained a yield of lipid by ether extraction of bacillus of Calmette-Guerin amounting to 23% of the R type and 11% of the S type, values calculated on the basis of total weight of bacteria. We obtained 24% of the Rv organism and 16.6% of the Ra organism as extractable lipoid. Cooper extracted twenty-five times with 300 cc. of ether at room temperature. In our experiment, the bacteria were subjected first to a continuous Soxhlet extraction,

(6) R. J. Anderson, R. Schoenheimer, J. A. Crowder and F. H. Stodola, *Z. physiol. Chem.*, **237**, 40 (1935).

using the solvents at a temperature just slightly below their respective boiling points, and were then extracted using the solvents at the boiling point. Furthermore, we used three solvents, ether, acetone and chloroform, where Cooper used only ether. These differences in experimental technique may account for the contradictory results.

Crowder, Stodola, Pangborn and Anderson[7] compared lipids obtained from four recently isolated strains of human tubercle bacilli with those from H-37. Qualitative results showed the same fatty acids in various strains; quantitative results showed great variation in lipid content. Total lipid content varied from 10.8 to 23.8%. Phosphatide content varied from 0.84 to 6.54%. Their results may explain our findings as being the usual variation observed in different lots of the same bacterium grown on the same medium; or the quantitative variations observed by them may be due to differences in proportions of rough and smooth forms in different lots of bacteria which would account for variations in lipoid content.

Sabin[8] in an extensive series of investigations has studied cellular reactions to the lipoids of the tubercle bacillus. This work shows that all of the lipoid fractions act as maturation factors for monocytes, epithelioid cells and giant cells resulting in the formation of tuberculous tissue. This effect was traced to peculiar fatty acids of high molecular weight isolated from the lipoids. It may be assumed therefore that the effect of the tubercle bacillus on the host can in some measure be attributed to the lipoid content of the bacteria.

Our results, showing a higher lipoid content of the virulent organism, suggest that virulence might be due to quantitative differences in lipoid content and/or mode of combination of lipoid in the cell. The variations in degree of virulence shown by the human H-37 tubercle bacillus again indicate that quantitative factors are concerned. If virulence depended entirely on qualitative factors, a clear-cut separation into virulent and avirulent organisms could be made. This is not possible in the case of the tubercle bacillus.

(7) J. A. Crowder, F. H. Stodola, M. C. Pangborn and R. J. Anderson, THIS JOURNAL, **58**, 636 (1936).

(8) Florence R. Sabin, Charles A. Doan and Claude E. Forkner, *J. Exptl. Med.*, **52**, Supplement No. 3, 1 (1930).

Summary

Marked differences in the lipoid contents of the dissociants of the human H-37 tubercle bacillus are shown by quantitative estimations of extractability with various solvents. The Rv form contains nearly twice as much lipoidal material which is more easily extractable by organic solvents. An analysis of media on which the two variants were cultivated showed differences in nitrogen utilization.

WINONA, MINN. RECEIVED JANUARY 31, 1938

[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY, UNIVERSITY OF WISCONSIN]

A Study of the Decomposition of Gaseous Ethyl Bromide

BY PAUL FUGASSI[1] AND FARRINGTON DANIELS

The decomposition of ethyl bromide has been studied previously in this Laboratory.[2,3] The present work was undertaken to determine whether theories of free radical chains are applicable to this reaction and to explore further the peculiar behavior of inert, foreign gases in decreasing the reaction rate. Particular attention was paid to the elimination of traces of oxygen and to the analysis of the products.

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(2) Lessig, *J. Phys. Chem.*, **36**, 2335 (1932).

(3) (a) Vernon and Daniels, THIS JOURNAL, **54**, 2563 (1932). (b) Vernon and Daniels, *ibid.*, **55**, 922 (1933).

Experimental Procedure

The molten lead thermostat was the same as used before except that the two stage amplifier was replaced with a single stage amplifier using a 2A5 tube. All the measurements reported here were carried out at 395 ± 0.15°.

The ethyl bromide was prepared by Professor Timmermans in Brussels and obtained from the U. S. Bureau of Standards.

Static Experiments.—Measurements were made in all-glass apparatus. Two different techniques were employed for filling the reaction chambers which were 125-cc. Pyrex flasks provided with glass diaphragms for measuring pressures.

The filling apparatus for the first procedure is shown in Fig. 1,