It has been shown that such compounds are represented by the formula Pb_2X_6 in pure state or in concentrated solutions, and by PbX_3 in very dilute benzene solutions.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY]

THE SUGAR CONTAINED IN TUBERCULINIC ACID, THE NUCLEIC ACID OF TUBERCLE BACILLI

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In previous papers by the authors[2] a description has been given of the method used for separating tuberculinic acid from tubercle bacilli, and also the nature of the pyrimidines which function in this acid. On the basis of new data obtained in our research we concluded that tuberculinic acid containing the pyrimidines, thymine and cytosine, but no uracil, resembles in its chemical characteristics a nucleic acid of animal origin, thereby establishing the natural group to which the tubercle bacillus is to be assigned. It is also known that the carbohydrates contained in plant and animal nucleic acids are characteristic of each group and differ in the two acids, hexose occurring in the acid of animal origin and pentose in the plant variety. Hence, an accurate knowledge of the sugar contained in tuberculinic acid would serve as a check on the above classification of the tubercle bacillus which is based on the results of a pyrimidine analysis.

The literature of the chemistry of tubercle bacillus reveals the fact that the carbohydrate phase of the work has received little attention at the hands of previous investigators, notwithstanding the fact that one of the first of them, Hammerschlag[3] recognized its importance. He was able to detect a reducing sugar in defatted bacilli, but thought he was dealing with cellulose. De Schweinitz and Dorset[4] were of the same opinion in regard to the nature of the carbohydrate contained in this material. Levene[5] isolated a carbohydrate of a glycogen-like nature as an impurity of the nucleic acid from tubercle bacilli, but did not further investigate it beyond the point of ascertaining that it formed a soluble copper salt which permitted its separation from the insoluble copper nucleate.

Bendix[6] was the first investigator to report the finding of a sugar complex in the nucleoprotein obtained from the tubercle bacillus. He characterized his product as a pentose, but gave no details as to the method employed in its isolation, nor any characteristic chemical tests in support of his conclusion. The only other investigator, so far

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[2] Brown and Johnson, *J. Biol. Chem.*, **54**, 721; 731 (1922).

[3] Hammerschlag, *Monatsh.*, **10**, 9 (1892).

[4] De Schweinitz and Dorset, THIS JOURNAL, **17**, 605 (1895).

[5] Levene, *J. Exp. Med.*, **6**, 135 (1901).

[6] Bendix, *Deut. med. Wochschr.*, **1901**, 16.

as the writers are aware, to report the finding of a sugar in the nucleoprotein from the tubercle bacillus was Goris[7] who claims to have found a hexose. The nucleoprotein was obtained from the defatted bacilli by extraction with water and precipitation with alcohol. Chemical data supporting this conclusion are also lacking.

Two other investigators working on tubercle bacilli have found sugars in the extracts of different solvents used on this material. Kozniewski[8] found that acetone extracted a substance from tubercle bacilli which, when treated with dil. mineral acids was decomposed with the formation of an inactive reducing sugar. Agulhon and Frouin[9] by the use of alcohol were able to extract a substance that yielded dextrose. These findings are of interest in showing the different parts of the bacillus from which the presence of sugars has been reported.

It is readily seen that the previous work on the carbohydrates of the tubercle bacillus gives very little accurate information in regard to the nature of the sugar functioning in this organism. Beyond the fact that a few investigators were able to find evidence of the presence of reducing sugars in different fractions of the tubercle bacillus, no conclusive results on the nature of these products have been obtained. Since the methods used for the identification of the different products were of a general nature and the material very complex, naturally the results obtained by the different investigators were incomplete and contradictory.

As a part of their investigation of the "Chemistry of the Tubercle Bacillus," which has been in progress in this Laboratory during the past 2 years, the writers have now been able to investigate the nature of the sugar contained in tuberculinic acid. After subjecting this nucleic acid to all the purification processes commonly used for the purification of other nucleic acids, a product was finally obtained which showed a constant content of nitrogen and phosphorus. Small portions of this material were used to ascertain qualitatively the nature of the decomposition products of the sugar, when subjected to acid hydrolysis. These tests showed that relatively large amounts of both formic and levulinic acids were produced and only very small amounts of furfuraldehyde. With this information in hand, greater amounts of tuberculinic acid were employed and the above products isolated and identified. Since hexose sugars yield on acid hydrolysis the above decomposition products and the pentoses under the same conditions yield only furfuraldehyde, the sugar functioning in tuberculinic acid is therefore definitely shown to be a hexose. The new data obtained by us are in complete accord with the results of our former work in which we found that thymine and cytosine were the only pyrimidines contained in tuberculinic acid, and show conclusively that the decomposition products of this nucleic acid are identical with those of known acids of animal origin, and different from those of the plant type in both their pyrimidine and sugar content.

[7] Goris and Liot, *Ann. inst. Pasteur,* **34,** 497 (1920).

[8] Kozniewski, *Bull. intern. acad. Sci. Cracovie,* Series A, p. 942, **1912.**

[9] Agulhon and Frouin, *Bull. soc. chim. biol.,* **1,** 176 (1919).

In the experimental part of this paper is given a complete description of the behavior of tuberculinic acid when subjected to hydrolysis, and a report of our examination of the hydrolysis products produced by the decomposition of the sugar.

Experimental Part

The Purification of Tuberculinic Acid.—The nucleic acid used in this work was separated from tubercle bacilli by the application of our method, described in our previous publications[2] to a large amount of human and bovine tubercle bacilli which had previously been defatted with toluene.

The crude tuberculinic acid, 8.4 g. obtained from 810 g. of dry tubercle bacilli, was further purified by solution in 1% sodium hydroxide solution, addition of acetic acid until the solution was neutral, then picric acid as long as a precipitate was formed and finally acetic acid until the mixture was distinctly acid to litmus. To the filtered solution hydrochloric acid was then added until a faint turbidity was produced and the nucleic acid precipitated from the solution by the addition of twice its volume of 95% alcohol. The precipitate was separated from the solution by a high-speed centrifuge, washed with 50% alcohol containing hydrochloric acid and finally with 95% alcohol, absolute alcohol and with anhydrous ether. The dry product was a light gray powder and contained 8.11% of phosphorus and 11.3% of nitrogen. This material was then dissolved in dil. potassium hydroxide solution, the solution poured into 6 times its volume of absolute ethyl alcohol, and the precipitated potassium salt separated by means of the centrifuge. This was then washed with absolute alcohol and dried with ether. A granular powder, slightly yellow in color, was obtained. The salt was again dissolved in water and converted into the copper salt by acidification with acetic acid, filtration, addition of hydrochloric acid until an opalescence was produced and, finally, addition of 10% copper chloride solution. The copper salt was purified and converted into the free nucleic acid according to the directions of Levene,[10] using 5% hydrochloric acid. Analysis showed that the percentages of nitrogen and phosphorus had not changed and that we had a product which further treatment did not alter.

The Decomposition Products of the Sugar Produced on Hydrolysis of Tuberculinic Acid with Hydrochloric Acid.—One g. of tuberculinic acid was placed in a 500cc. distilling flask with 250 cc. of hydrochloric acid (d., 1.06) and the mixture distilled from an oil-bath at 150°. At intervals, when 25 cc. of distillate had passed over, an equal volume of hydrochloric acid (d., 1.06) was added maintaining approximately the same strength of solution. The distillation was continued for 7 hours, during which about a liter of distillate was collected. The solution did not boil smoothly, but occasionally foamed and super-heated, and on one occasion boiled over, a few drops reaching the bulk of the distillate before the receiver could be changed.

To the distillate an excess of phloroglucine dissolved in hydrochloric acid was added and the mixture heated at 80° to 85° for 2 hours and then allowed to stand overnight. The phloroglucide precipitate was collected on an asbestos mat in a Gooch crucible that had been previously dried to constant weight at 98°, washed with water and again dried to constant weight at the same temperature. This weighed 0.024 g. Since Osborn and Harris[11] under the same conditions obtained 0.252 g. of this product from 1 g. of tritico-nucleic acid from wheat, it is readily seen that the 2 acids differ markedly from each other in the amount of furfuraldehyde produced on hydrolysis. Steudel,[12] working

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[10] Levene, *J. Biol. Chem.*, **48,** 177 (1921).

[11] Osborn and Harris, *Z. physiol. Chem.*, **36,** 85 (1902).

[12] Steudel, *ibid.*, **56,** 212 (1908).

with thymus nucleic acid, also obtained furfuraldehyde, but in amounts somewhat smaller than the quantity produced from tuberculinic acid. The results obtained in the case of tuberculinic acid agree very closely with the results obtained by other investigators working with animal nucleic acids and differ materially from the results obtained when the plant acids were examined.

The solution remaining from the preceding experiment was utilized to ascertain whether levulinic acid was produced as a hydrolytic product of tuberculinic acid. The distillation was continued to dryness and the residue exhaustively extracted with ether, the ether extract poured through a dry filter paper and the ether then expelled by distillation. The gummy residue was heated on the hot plate for 2 hours to expel any hydrochloric or formic acids that it might contain, dissolved in 25 cc. of water and finally filtered from a small amount of insoluble material. Preliminary tests on 1cc. portions of this solution showed that it gave a strong iodoform reaction, as well as a deep red color when sodium nitroprusside and a few drops of sodium hydroxide solution were added, the color changing to violet after the addition of an excess of acetic acid. These tests, especially the latter, are characteristic of levulinic acid. The remainder of the solution was digested for 1 hour with an excess of zinc oxide according to the directions of Wehmer and Tollens[13] in order to prepare the zinc salt. After decolorization with animal charcoal the filtered solution was evaporated to a small volume, when a precipitate began to separate; as the solution cooled, more was obtained. This precipitate was crystalline, showing 6-sided crystals when viewed under the microscope, and was identical in appearance with the zinc salt prepared from levulinic acid. The solution was evaporated to dryness, the residue washed with a mixture of equal parts of absolute alcohol and ether, dissolved in water and silver nitrate solution was added. A precipitate was obtained but in too small amount for a silver determination. These tests show conclusively that levulinic acid is produced on acid hydrolysis of tuberculinic acid.

The Isolation of Levulinic Acid from Tuberculinic Acid.—To 3.8 g. of tuberculinic acid in a 300cc. round-bottom Pyrex flask, 30 cc. of water and 10 cc. of concd. sulfuric acid were added and the mixture was heated in an oil-bath at 125° for 25 hours under a return condenser. At the end of this time 50 cc. of water was added and the solution distilled until 50 cc. had passed over. This distillate, which contained formic acid was saved, as described below.

The solution remaining in the hydrolysis flask was exhaustively extracted with ether, the ether extract poured through a dry filter and the ether distilled on a water-bath. The residue was allowed to remain on the steam-bath to dispel traces of water and formic acid. A viscous sirup was obtained, weighing 0.5 g. and composed chiefly of levulinic acid. This amount figured in terms of its hexose equivalent corresponds to 37.5% of the weight of tuberculinic acid used. The crude residue was dissolved in water, exactly neutralized with sodium hydroxide solution, and silver nitrate solution added in excess. A beautiful precipitate of the silver salt of levulinic acid was obtained. This was recrystallized once from water and dried at 100°.

Analysis. Subs., 0.1923: Ag, 0.094. Calc. for $C_5H_7O_3Ag$: Ag, 48.43. Found: 48.88.

In other words, it was established that this compound was identical in every respect with the silver salt prepared from levulinic acid.

The Identification of Formic Acid.—The distillate obtained as described above was strongly acid to litmus, and when a few cubic centimeters was warmed with silver nitrate solution a silver mirror was obtained. A black deposit of mercury was immediately obtained when a crystal of mercurous nitrate was added and the solution warmed. A portion of the solution when neutralized with sodium hydroxide and treated with ferric chloride solution gave the characteristic red color when boiled, showing the formation

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[13] Wehmer and Tollens, *Ann.*, **243,** 314 (1887).

of ferric formate. When 30 cc. of the distillate was neutralized with sodium hydroxide the solution, then made acid to litmus with hydrochloric acid, and a solution of mercuric chloride added and the mixture digested on the water-bath, a white precipitate soon began to form. A large amount of calomel was obtained, covering the entire bottom of the beaker. These results show conclusively that the acid contained in the distillate was formic acid.

The Quantitative Determination of the Pyrimidines.—The solution remaining from the ether extraction was worked for the pyrimidines according to the directions given in one of our previous papers.[2] After the removal of sulfuric acid with barium hydroxide, and the purines with silver sulfate in acid solution, the pyrimidines were precipitated in the usual manner as silver salts. These salts were decomposed with hydrogen sulfide and the cytosine removed as the phosphotungstate. After decomposition of this salt with barium hydroxide the cytosine was isolated as the base; 0.37 g. was obtained, corresponding to 9.7% of the weight of nucleic acid used.

The filtrate left from the phosphotungstate precipitation was freed from this reagent with barium hydroxide, the barium removed in the usual way and the solution evaporated to dryness. The residue was taken up in water and filtered from a small amount of insoluble material and the solution again evaporated to dryness and weighed. The crude thymine weighed 0.45 g., corresponding to 11.8% of the weight of the nucleic acid used. All of our solutions failed to respond to tests for uracil.

Summary

1. It has been shown that the sugar functioning in tuberculinic acid is a hexose. This was proven by identification of levulinic and formic acids as products of its hydrolysis.

2. A new analysis for pyrimidines in tuberculinic acid has been made and results have been obtained which are identical with those reported in our previous publication.[2]

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[Contribution from the Chemical Laboratory of the University of California]

THE ACTION OF ULTRAVIOLET LIGHT UPON DIKETONES

By C. W. Porter, H. C. Ramsperger and Carolyn Steel

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In aqueous solution benzil is converted into benzilic acid by warming it with an alkali. The reaction is rapid and practically quantitative. It has been reported that the same rearrangement occurs slowly in the absence of alkali when benzil, in moist ether, is exposed to sunlight.[1] Under similar treatment benzil in alcohol yields benzoic acid and benzoin.[2]

This investigation was undertaken to determine the action of radiant energy of short wave lengths upon aqueous and alcoholic solutions of benzil and the corresponding aliphatic diketone, diacetyl.

Preliminary tests were made with saturated aqueous solutions of benzil. The solutions were placed in open beakers with the liquid surfaces 18 cm. below a 110-volt quartz mercury-vapor lamp. Samples were withdrawn

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[1] Klinger, *Ber.*, **19**, 1868 (1886). Lachman, This Journal, **44**, 330 (1922).

[2] Ciamician and Silber, *Ber.*, **36**, 1575 (1903).