The high nitrogen values for the washings are due to non-antibody protein of the serum. The progressive reduction in the values indicates the removal of this protein by successive dilution. The possibility that non-antibody protein was non-specifically taken up in the lattice of agglutinated ghosts cannot be excluded. Therefore a portion of the nitrogen in the resuspended antibody may not represent antibody nitrogen.

Summary. Study of a human cold hemagglutinin revealed:

1. The cold hemagglutinin had the electrophoretic mobility of gamma globulin. 2. A cold hemagglutinin titer of 1/2560 at 4°C was equivalent to 1.473 mg per ml of antibody nitrogen.

14244

Metabolism of Sulfapyridine in the Dog.

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The therapeutic efficacy of the sulfonamides has stimulated an interest in the possible changes these compounds may undergo in the animal body. It is well known that the sulfonamides are acetylated in the human body and in this form they are relatively inactive. However, it is possible that other forms of conjugation or alteration in structure may occur which might modify their toxicity or activity.

Scudi[1] reported the isolation from dog urine of an hydroxysulfapyridine conjugated with glucuronic acid following the administration of sulfapyridine. No details were given on the isolation of this compound nor data concerning the probable position of the hydroxy group. Thorpe, Williams, and Shelswell[2] have suggested the possibility that the hydroxyl group might be introduced in the animal body in either the 2 or 3 position of the benzene ring of the sulfonamide.

Our interest in this problem was aroused when we were unable to isolate from the urine sulfapyridine as the reduction product of 2-(p-nitrobenzenesulfonamido) pyridine when the latter compound was administered orally to dogs. The same difficulty was encountered when sulfapyridine was administered. Sulfapyridine added to urine can be isolated readily by prolonged ether extraction. We therefore, decided to attempt the isolation of the diazo reacting substances which are excreted in dog urine when sulfapyridine is administered orally. The values given for diazo reacting substances in the urine are those determined by the Bratton and Marshall[3] method, using sulfapyridine as a standard.

Four dogs were given orally 5 g of sulfapyridine daily for 4 days and the urines were collected daily for 6 days, and preserved with chloroform. The combined urines gave a total sulfapyridine value of 67 g and no increase in color was obtained after acid hydrolysis indicative of the fact that the dog apparently does not conjugate any of the sulfapyridine as an acetyl derivative. Prolonged ether extraction in a continuous extractor for 9 hr resulted in the extraction of 9 g of diazotizable material as determined colorimetrically. When small quantities were extracted with ether in a more efficient extractor, 16.5% of the total diazotizable material was extracted by ether and 13.8% was extracted in the first 3 hours. This value of 16.5% probably represents the total amount of sulfapyridine which is excreted unconjugated. The gummy material extracted with

[1] Scudi, J. V. *Science*, 1940, **91**, 486.

[2] Thorpe, W. V., Williams, R. T., and Shelswell, *Biochem. J.*, 1941, **35**, 52.

[3] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

ether yielded a total of 2 g of crystalline material which melted at 190-191°C and no depression of the melting point was produced when mixed with sulfapyridine. The nitrogen content (Kjeldahl) was 16.5% (calculated for sulfapyridine 16.8%).

The urine extracted with ether was treated with an excess of lead acetate and the mixture acidified with acetic acid. The heavy precipitate obtained was filtered off. The filtrate was made alkaline with sodium hydroxide (10%) and the precipitate of lead hydroxide obtained, was filtered off and the filtrate discarded. The lead precipitate was suspended in water and the lead removed with hydrogen sulfide. The filtrate from the lead sulfide containing 45 g of diazotizable material or 67% of that present in the original urine. A portion of this filtrate containing the equivalent of 9 g sulfapyridine was treated with silver nitrate which produced a heavy flocculent precipitate. This precipitate was filtered off and resuspended in boiling water which dissolved the greater part and from which it would again precipitate on cooling. This precipitate was collected and dried *in vacuo*. On analysis it showed a silver content of 29.6%, nitrogen content of 6.45% and a color value equivalent to 35.2% sulfapyridine. On hydrolysis with N HCl the solution reduced Benedict's solution and the glucose equivalent was 40.1%. This compound is probably the silver salt of a glycuronic acid conjugate of a sulfapyridine derivative, which has been described by Scudi,[1] although we were never able to isolate a crystalline product or one that gave a constant silver value, the silver contents varying from 26.7% to 29.5%.

The remainder of the lead free filtrate was evaporated to 1000 cc and 100 g sodium hydroxide was added and mixture refluxed for 5 hours. This hydrolyzed solution was neutralized to approximately pH 6.5 and extracted with ether in a continuous extractor for 24 hours. During the ether extraction a crystalline product separated from the ether. A total of 26.2 g of crystalline material was obtained. This was dissolved in acetone by addition of water. The material was light pink and on drying *in vacuo* lost its crystalline appearance and approximately 10% in weight. It gave a positive diazo reaction and gave a color equivalent to sulfapyridine of 89%. Ferric chloride gave a deep purple color which changed rapidly to dark brown. It melted at 190°C and a depression of the melting point was produced when mixed with sulfapyridine. The nitrogen content was 15.6%.

14 g of the above compound was refluxed with 100 cc 10% hydrochloric acid for three hours. The material went completely in solution but after 2 hours of boiling a crystalline material began to precipitate. The mixture was cooled overnight and the crystalline material separated and dried. After recrystallization from water 5.2 g of crystalline material was obtained. This material gave a positive diazo reaction and gave a color value equivalent to that given by sulfanilic acid. The $FeCl_3$ test was negative. The M.P. was above 300°C. The nitrogen content (Kjeldahl) was 7.91%, calculated for sulfanilic acid 8.09%.

The acid filtrate from the above crystals was treated with an excess of picric acid. The copious precipitate was filtered off and dried. Recrystallization from water gave prism-shaped crystals which melted at 212° and the melting point was depressed when mixed with 2-amino-pyridine picrate. The picrate was suspended in water acidified with HCl and the picric acid removed with ether. The extracted solution was evaporated to dryness and the residue readily dissolved in 15 cc absolute alcohol. The cautious addition of ether resulted in the appearance of crystals and then 10 volumes of ether was added. On recrystallization, 2.85 g of crystalline material was obtained. It gave a strong positive $FeCl_3$ test, negative diazo test, and melted at 126°C. Analysis: Cl-23.7%; Nitrogen (Kjeldahl) 18.7%. Calculated for 2-amino-hydroxy-pyridine hydrochloride—Cl 23.5%, nitrogen 19.11%. Our data indicate that sulfapyridine is excreted in dog urine largely combined with a reducing substance presumably glucuronic acid. Alkaline hydrolysis of this compound results in the liberation of a compound giving a positive ferric chloride test indicative of the presence of an hydroxyl group. Acid hydrolysis of this compound permitted the isolation of sulfanilic acid and a pyridine residue which gives a positive

ferric chloride test. Therefore the dog apparently metabolizes sulfapyridine in greater part by introducing an hydroxyl group in the pyridine ring and then conjugating with a carbohydrate. This hydroxy derivative possesses some therapeutic effect in mice injected with hemolytic streptococcus and produces bacteriostasis *in vitro*, but the efficacy is less than that of sulfapyridine. As much as 80% of the diazo reacting compounds appearing in dog urine following the ingestion of sulfapyridine is in the form of this glycuronide. Only a small portion which probably is not greater than 16 percent is excreted as sulfapyridine. At least in the dog no hydroxyl group is introduced in the benzene ring and this is further verified by the fact that sulfanilamide is excreted without change.

Summary. The ingestion of sulfapyridine in the dog is followed by the appearance in the urine of a glycuronide of a hydroxy derivative of sulfapyridine. The hydroxy group is attached to the pyridine ring.

14245 P

Enhancement with Phenol of the Serological Reactivity of Lymphogranuloma Venereum Antigens.

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Three types of complement-fixing antigen prepared from yolk sac cultures of the agent of lymphogranuloma venereum have been described; *viz.*, (1) resuspended high speed sediment,[1] (2) soluble antigen in high speed supernates,[2] (3) whole suspensions inactivated with urea or ether.[3] The third type was approximately four times as active as high speed sediment.

Attempts by one of the authors (Nigg) to find a satisfactory preservative for lymphogranuloma venereum complement-fixing antigens, showed that phenol enhanced the activity of the antigen specifically since such phenolized antigens did not react with normal sera. This observation was studied further and is the subject of this note.

Table I shows the complement-fixing activity of a 10% yolk sac suspension treated with (a) 2% urea, (b) 0.25% phenol and (c) 0.5% phenol. Preparations were stored at icebox temperatures. Two separate fractions of suspension 56 when treated with 0.25% phenol failed to show increased activity when tested 8 to 30 days later. Two fractions treated with 0.5% phenol were 4 and 8 times respectively as active as the urea-treated antigen.

While 0.25% phenol had little enhancing effect at icebox temperatures, Table II shows approximately a 4-fold increase in activity when these suspensions were subjected to the following temperatures: (a) 37°C for 6 weeks (not tested earlier), (b) 56°C for 48 hours, and (c) boiling water for 10 minutes. Furthermore, the centrifuged (2,000 rpm for 10 minutes) supernates of the heated suspensions were almost as active as the whole heated suspensions. This represents a considerable purification since the slightly opalescent supernates, after removal of tissue debris and coagulated protein, were almost as active as the whole heated turbid suspensions.

That the phenol enhancement is apparently specific is shown in diagnostic tests (Table III) with positive and negative sera, comparing 2 phenolized antigens with urea-treated antigen. Antigen 57-H was treated

[1] McKee, C. M., Rake, G., and Shaffer, M. F., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 410.

[2] Rake, G., Shaffer, M. F., Jones, H. P., and McKee, C. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 300.

[3] Nigg, C., PROC. SOC. EXP. BIOL. AND MED., **1942, 49, 132.**