product gave a reddish-brown coloration with iodine which is typical of glycogen.

The spectra of whole cells can now be used to follow the course of glycogen production and destruction under various conditions of growth. The amount of this polysaccharide in the bacterial cell can be estimated from the intensity of the 9.75-μ glycogen band. This is compared to the 6.45-μ protein absorption band which acts as an internal standard. The method has the following limitations: the absorption of small amounts of glycogen may be masked by bands due to other materials, particularly slime-layer or capsular polysaccharides; and the spectrum of glycogen resembles closely the spectrum of starch or dextrin. The advantages of the method are that it is rapid and simple, it involves no chemical manipulations, and it requires very small samples (as little as 0.4 mg of dried cells) (*2*). Later observations on the occurrence of glycogen in bacteria, as well as chemical and ultracentrifugal studies of this polysaccharide, will be reported.

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Micro Determination of Isoniazids in Blood

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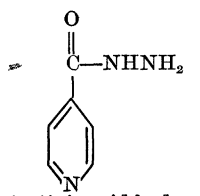
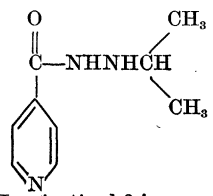
Compounds like isonicotinic acid hydrazide and the isopropyl derivative of isonicotinic acid hydrazide (1-isonicotinyl-2-isopropyl hydrazine) have been approved for use in the treatment of tuberculosis under medical supervision by the U.S. Food and Drug Administration. Accurate and sensitive methods for the determination of these compounds in blood would be valuable in following the course of treatment. Such methods have been proposed by Rubin *et al.* (*1*) and by Kelly and Poet (*2*). These methods are, however, cumbersome and do not lend themselves readily to the ordinary techniques of a clinical laboratory. The monaqueous titration method of Alicino (*3*) and the iodometric methods of Canbäck (*4*) and of Alicino are not applicable.



Isonicotinic acid hydrazide



1-Isonicotinyl-2-isopropyl hydrazine

The following method is simple and more rapid than the methods mentioned. It depends upon the reduction of potassium ferricyanide in acid solution by the isoniazid with the subsequent formation of a Prussian or Turnbull's blue and the colorimetric estimation of the greenish color formed from the yellowish of the ferricyanide and the blue of the iron complex compound.

Reagents. Sodium tungstate solution, 10%: Dissolve 10 g of reagent grade, carbonate-free sodium tungstate, $Na_2WO_4 \cdot 2H_2O$ in water and dilute to 100 ml.

Sulfuric acid, 2/3*N*: Tare a 50-ml beaker. Weigh into this beaker 35 g of concentrated sulfuric acid, specific gravity 1.84. Place approximately 500 ml of distilled water into a liter volumetric flask. Pour the sulfuric acid carefully into the liter flask containing the water, while swirling the flask. Cool, complete to volume, and check the normality against standard sodium hydroxide solution. Adjust the concentration, if necessary.

Acetic acid 3*N*: Prepare in the customary manner.

Potassium ferricyanide solution: Dissolve 2 g of analytical, reagent grade potassium ferricyanide, $K_3Fe(CN)_6$, in distilled water and dilute to 500 ml. Store in a brown bottle protected from light and preferably under refrigeration. Transfer small volumes to another brown bottle for daily use or prepare a fresh solution, as desired.

Standards may be conveniently prepared using recrystallized 1-isonicotinyl-2-isopropyl hydrazine phosphate or isonicotinic acid hydrazide and normal horse serum or pooled normal human blood plasma.

Standard isoniazid solutions: Weigh carefully 0.200 g of isoniazid and transfer to a 200-ml volumetric flask. Add distilled water, shake until dissolved, and dilute to volume. This is the stock standard solution and contains 1 mg/ml.

Transfer with the aid of a volumetric pipet 1 ml of the stock standard solution to a 50-ml volumetric flask and dilute to volume with distilled water. This solution contains 20 μg/ml.

Standard curves: Transfer with the aid of pipets 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 ml of the diluted standard isoniazid solution containing respectively, 10, 20, 30, 40, 50, 60, and 80 μg of isoniazid to 1 ml of normal horse serum or pooled normal human blood plasma. Dilute to 6 ml with distilled water and proceed with the method as detailed below. Since only 4 ml of the tungstate filtrate is used, the standard curve represents half the concentrations listed above, namely, 5, 10, 15, 20, 25, 30, 35, and 40 μg, respectively.

Transfer 1 ml of blood plasma to a 16 × 150-mm test tube. Add 5 ml of water, mix; add 1.0 ml of 10% sodium tungstate solution and mix with an individual glass rod; add 1.0 ml of 2/3*N* sulfuric acid, mix with an individual glass rod, and place the tubes in a water bath at 80–85° C for 1 min to coagulate the precipitate. Remove the tubes from the hot bath, chill in a cold water bath, dry the outside of the tubes, and filter

through a 9-cm Schleicher and Schuell No. 576 filter paper.

Transfer 4 ml of the clear filtrate to a Klett-Summerson colorimeter tube or other tube, add 0.5 ml of 3*N* acetic acid, and mix by swirling; add 0.5 ml of potassium ferricyanide solution, and mix again by swirling. Place the tubes in a hot water bath at 80° C for 15 min. Remove the tubes from the bath, cool under running water, or place momentarily in an ice bath and read 25 min after initial immersion in the heating bath, in a Klett-Summerson photoelectric colorimeter using a No. 66 filter. Compare against standards treated similarly at the same time or use a prepared curve. Multiply the result by 2 to get the micrograms of isoniazid per milliliter of blood plasma.

The method was tried using human blood plasma[1] and serum, normal horse plasma,[2] and normal sheep plasma because these were available. Over 150 such samples were tested and all were negative or virtually so under the conditions of the test.

Known volumes of standard solutions of the purified phosphate of the isopropyl derivative of isonicotinic acid hydrazide (1-isonicotinyl-2-isopropyl hydrazine phosphate), Marsilid[3] phosphate, and of purified isonicotinic acid hydrazide, Rimifon,[3] were added before and after tungstate-acid treatment.

Representative results obtained by use of this method with known amounts of added Marsilid phosphate to blood plasma samples are, in micrograms and Klett-Summerson scale readings respectively: 0, 0; 5, 21; 10, 37; 15, 42; 20, 64; and 25, 98. These results indicate that the method can be used for the determination of isoniazid in blood in the order of 4–5 μg and up.

The theoretical aspects, accuracy, precision, and sensitivity of this method have been discussed in another paper (*5*). In that paper it was pointed out that the concentration of acid, the temperature, and the isoniazid in question have a pronounced effect on the sensitivity of the reaction. When the original method of assay, using a boiling water bath and hydrochloric acid for the ferricyanide reduction step, was applied to blood, occasional samples of human plasma known not to contain isoniazids, reduced the ferricyanide. This interference was eliminated when hydrochloric acid was present by increasing the acid concentration of the final reaction mixture from approximately 0.1*N* to approximately 0.3*N* and by reducing the temperature of heating from 99–100° C to 79–80° C. The reduction in heating temperature necessitated increasing the heating period from 5 min to 15 min. The heating period at 80 ± 0.5° C should be kept to 15 min. The overall heating time from 75 to 80° C may be 20 min. When acetic acid was used, however, the reduction could be carried out at 100° C.

The reduction of ferricyanide as a method of determination has been applied in various ways, possibly the best known clinical application is that of the determination of glucose (*6–8*). The glucose method, however, is performed in alkaline solution generally in the presence of cyanide-carbonate solution. The ferricyanide method detailed here is performed in an acid solution. Under these conditions glucose does not interfere nor do the normal components of blood.

Poor results were obtained when trichloroacetic acid was used as the protein precipitant. While better precipitation was obtained with hexametaphosphoric acid than with tungstic acid, the sequestering action of the hexametaphosphoric acid prevented the formation of the Prussian blue.

An attempt was made to adapt this method to the determination of isoniazids in urine but anomalous results were obtained even when the heating temperature was lowered to 65° C and the final acid concentration was increased to over 1*N*. Work is continuing on this variation.

Since the method described in this paper is an empirical method, one should adhere closely to the procedure as detailed, paying close attention to the acid concentration, the time of heating, prompt cooling, and prompt reading. The analyst may, however, vary conditions to suit his needs but then must prepare standard curves conforming to the changed conditions or must carry along standards using the changed conditions.

[1] The human blood plasma was obtained from samples submitted for typing to the Rh Laboratory of the Bureau of Laboratories, Dept. of Health of New York City.

[2] The normal horse plasma and normal sheep plasma were obtained from the Research and Antitoxin Laboratory of the Bureau of Laboratories of the Department of Health, City of New York, at Otisville, N. Y., where much of the work was performed.

[3] Trade name of Hoffmann-La Roche, Inc.

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