Summary. A safe, time-saving, closed-vacuum harvesting technic is described. The advantages and safeguards of this technic are enumerated.

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Improved Method for Determination of Plasma Polysaccharides with Tryptophan.* (20620)

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(Introduced by Morris Ziff.)

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There is currently an active interest in the determination of the polysaccharide content of plasma and other biological fluids as related to different diseases. The tryptophan reaction proposed by Shetlar, Foster, and Everett(1) has, for clinicians, certain advantages over the orcinol reaction of Sorensen and Haugard(2). The stock tryptophan solution can be kept for several days on ice; the production of the color at 100°C does not call for the greater care required in the orcinol reaction which must be run at 80°C; and the color produced is less affected by impurities in the reagents. As described, the tryptophan reaction has the serious failing of giving colored products whose absorption spectra differ widely with different sugars. For instance, glucose gives a yellowish-brown color while mannose gives a pure violet. It will be shown that when the reaction is carried out in the presence of boric acid this failing is diminished. The best results are obtained with the highest possible concentration of boric acid.

Reagents. 1) Absolute ethanol. 2) Boro-sulfuric acid, containing sulfuric acid (770 ml), water (230 ml) and boric acid (50 g). For comparison, a sulfuric acid solution is prepared omitting the boric acid. 3) Tryptophan (1 g) dissolved in boiling water (100 ml). The L-form is preferable to the DL-form because it is more soluble and solutions kept on ice do not crystallize. 4) Standard mannose solution (50 to 150 μg/ml). This can be kept on ice for several weeks in the presence of a few drops of toluene.

Procedure. The reaction is best carried out in carefully cleaned matched tubes that are to be used in the photometer. Serum (.05 ml) is introduced, and then absolute alcohol (about 10 ml). After 15 minutes the tube is centrifuged at 2500 rpm for 10 minutes, the alcohol is decanted, water (1 ml) is added and the precipitate is dispersed. From a burette, boro-sulfuric acid solution (7 ml) is added and the contents of the tube are immediately mixed and chilled in ice water for 5 minutes. After leaving the tube at room temperature for 15 minutes, the tryptophan solution (1 ml) is added. The contents of the tube must be mixed immediately. Because of the high density of the sulfuric acid solution this is best done by bubbling air through it. The tube is again chilled in ice water. At the same time a series of 6 or 8 tubes is similarly prepared containing different amounts of the standard mannose solution, each made up to 1 ml before addition of the boro-sulfuric acid. All the tubes are heated in a boiling water bath for 20 minutes, and the contents again mixed once or twice during this time. The tubes are then

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TABLE I. Comparison of Wave Lengths of Maximum Absorption and Optical Densities, at These Wave Lengths, Produced by Different Sugars in the Sulfuric Acid and the Boro-sulfuric Tryptophan Reactions.

Sugar	Wave length (mμ) of max. absorption		Optical density/100 μg sugar at max.	
	Sulfuric acid	Boro-sulfuric acid	Sulfuric acid	Boro-sulfuric acid
Glucuronic acid	480	540	.258	.393
Glucose	440	525	.504	.703
Arabinose	480	520	.526	.680
Galactose	480	520	.478	.586
Mannose	500	520	.586	.700
Fucose	500	520	.588	.646

chilled for 5 minutes in a cold water bath and left at room temperature for a half hour. Optical density is measured at 520 mμ or with a green filter. Some brands of sulfuric acid gave a marked yellow color when heated with the tryptophan solution. Only sulfuric acid that gives no more than a faint yellow tint should be used. The tryptophan solution, stored on ice, should not be kept for more than 10 days. If the tube contents are not mixed immediately after the addition of the tryptophan, a false yellow color develops in the zone where tryptophan and boro-sulfuric acid meet. In all work described, the optical density is corrected by subtraction of the optical density of an identically made solution containing no sugar. An advantage of the boro-sulfuric acid over the sulfuric acid reagent lies in the fact that this correction is smaller. In a 1 cm cell this correction is .192 at 425 mμ, .092 at 475 mμ, and .052 at 525 mμ.

Results. Six sugars were examined by the method described. In all cases the color produced in boro-sulfuric acid is a pure violet compared to the more variable brown to violet colors produced in sulfuric acid. For each sugar the plot of corrected optical density against the amount of sugar used is linear up to 150 μg. This is true in the absence as well as in the presence of boric acid. The data are not presented in detail but the slope of each of these lines is given in Table I as well as the wave length at which maximum optical density occurs. The spectra of the colors produced by each of the sugars in the absence and presence of boric acid are shown in Fig. 1. In this reaction glucosamine and galactosamine produce no color at all.

Since the main purpose of this work was to provide a reliable and quick assay method for the polysaccharides in plasma it became necessary to ascertain whether the presence of boric acid would interfere with the hydrolysis of these polysaccharides or the rate of color development. That boric acid does not interfere with either process is shown in the curves of Fig. 2.

The thought of Sheppard and Everett(3) underlying the development of the reaction of sugars with tryptophan seems to have been that the color was due to the formation of black humin from furfural and tryptophan. This idea was tested by observing the color developed with furfural and tryptophan in the absence and in the presence of boric acid. For this purpose furfural was freshly distilled and a solution made containing 100 μg per ml. Its behavior was the same in the absence or presence of boric acid; a bright yellow color is produced instantly in the cold on the addition of tryptophan, and this is converted after 20 minutes of heating at 100°C to a reddish-brown color. No trace of violet color is produced as occurs with all the sugars tried. It must be concluded that the violet color is not a result of furfural formation from any of the sugars. This was true even of glucuronic acid which does yield furfural on heating with sulfuric acid. If glucuronic acid is first heated with either the sulfuric or boro-sulfuric solution used in the test and then cooled, addition of tryptophan produces the same yellow color as with furfural and only a brown color develops on heating at 100°C. Experiments

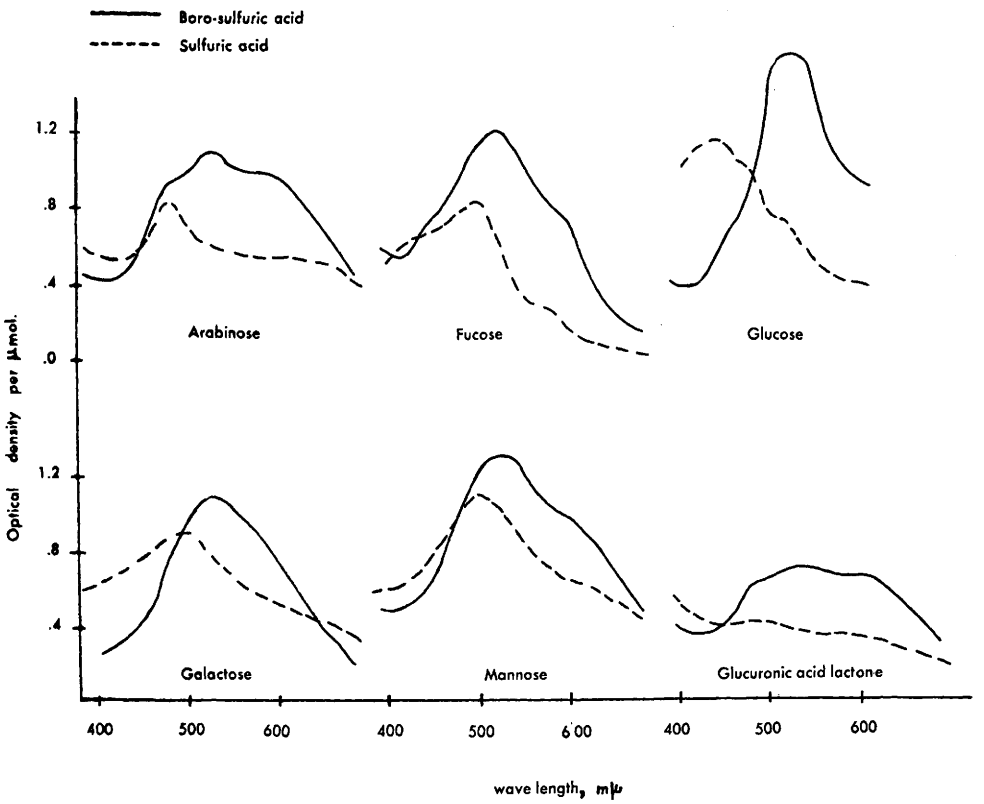

FIG. 1. Comparison of spectra of colored products resulting from tryptophan reaction in sulfuric acid solution and in boro-sulfuric acid solution.

were also carried out using a 1% solution of indole in 50% ethanol instead of the tryptophan solution. Only brown colors were produced which in a few cases were intensified by the presence of boric acid.

It has also been found that if the colors are developed by allowing the reaction mixtures to stand for 24 hours at 37°C instead of heating for 20 minutes at 100°C, then all the sugars develop violet colors with maxima at 520 to 540 mμ even in the absence of boric acid. Under these conditions the presence of boric acid also produces a higher color density.

Discussion. The advantages of this new tryptophan method are that there is an increase in optical density for each sugar, that the maximum optical density occurs at longer wave lengths where the blank correction is smaller, that the wave lengths of optical density are more nearly the same for different sugars, and that there is a small decrease in the difference in color density produced by different sugars.

The striking effect of boric acid on the color produced by the reaction of tryptophan with sugars might be associated with the ability of boric acid to form complexes with sugars and to protect them from side reactions with the sulfuric acid. But in acid medium boric acid seems not to form complexes with sugars according to work of Rumbach and Weber(4), Boeseken(5), and Levy and Doisy(6). In alkaline solution Levy and Doisy(7) found that glucose was protected against oxidation by alkaline hypoiodite to a greater extent than arabinose and galactose. This is the same as the order found in the present work for the extent of the effect of boric acid on the color produced in the tryptophan reaction.

Because of the absence of evidence for a reaction between boric acid and sugar in the acid medium, another thought was considered to account for the improvement in color it produced. Boric acid reacts with sulfuric acid

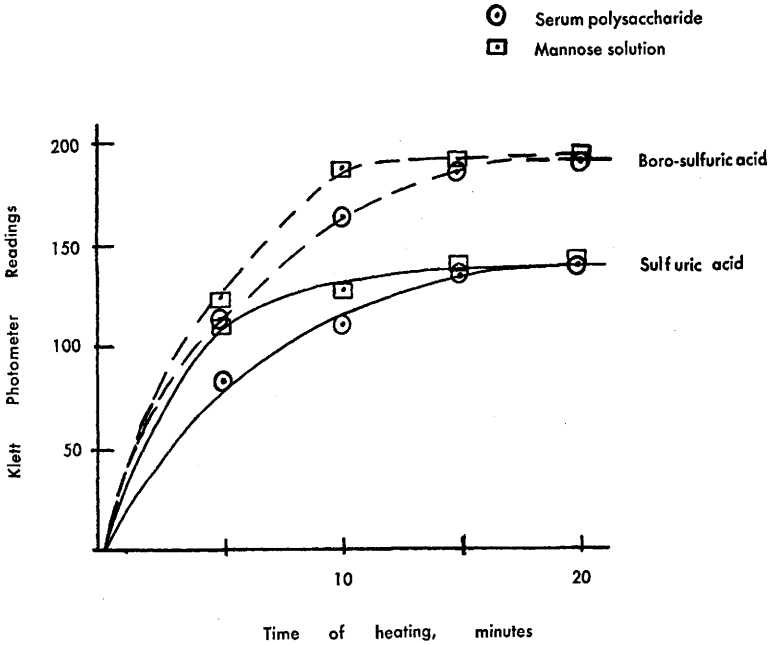

FIG. 2. Comparison of rate of color development in a serum sample and in a standard mannose solution when the tryptophan reaction is run in sulfuric acid solution and in boro-sulfuric acid solution. Protein from .05 ml normal serum was precipitated with ethanol (10 ml) and the tryptophan reaction was carried out on this precipitate. For comparison, the reaction was also carried out on 45 μg of mannose which yields the same final optical density. The color develops slightly more slowly in the serum due to progressive liberation of sugars, but this is not influenced by boric acid.

to produce borylsulfates. This raised the question whether borylphosphates or borylacetates might not also bring about such an improvement. Trial of a variety of mixtures of phosphoric or acetic acids with sulfuric acid and with or without boric acid, however, yielded no improvement in color.

Summary. A method to measure the concentration of sugars in solution, particularly of polysaccharides in biological fluids such as plasma, is presented. It is a modification of a tryptophan method already described and utilizes boric acid in the reaction medium. The color intensity is increased and the color quality modified to give maximum absorption at a longer wave length where interference by non-specific brown produced by reagent blanks and proteins is diminished.

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