CX. THE DETERMINATION OF FRUCTOSE IN BLOOD

By FREDA KATHARINE HERBERT

*From the Pathological Department of the Medical School, King's College,
and the Royal Victoria Infirmary, Newcastle-upon-Tyne*

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THE estimation of blood fructose has recently found a place in clinical work. It has been shown that in estimating fructose tolerance as a test of hepatic function, determinations of blood fructose are much more satisfactory than determinations of total blood sugar. Results based on blood fructose estimations have been reported by Scott [1935, 1], who used his own method of analysis [Scott, 1935, 2], by Stewart *et al.* [1937], who used a modification of the method of Patterson [1935], and by Herbert & Davison [1938], who used Patterson's method.

In the course of work with the diphenylamine method as used by Patterson, certain defects in the method became apparent, and modifications were therefore made which give greater accuracy and also greater speed and simplicity in working.

With Patterson's method, the writer found that irregularities sometimes occurred, both in analyses of pure fructose solutions and in the recovery of fructose added to blood. The error was seldom sufficient to affect the clinical interpretation of results, but greater accuracy was desirable. Another difficulty arose in the analysis of blood taken in the fasting state. Such samples always gave a slight greenish blue colour. This could not be accurately estimated, partly because of its faintness, and partly because the tint was different from that obtained with pure fructose; but approximate estimations of the intensity of the colour gave figures ranging from 3 to 7 mg. per 100 ml., expressed as fructose, in normal subjects. Experiments with fructose added to blood showed that the colour derived from blood alone could not be ignored, but had to be taken into account in order to obtain consistent results for the recovery of added fructose. These observations are in conflict with Patterson's results for fructose added to blood [Patterson, 1935]. Glucose reacts with diphenylamine under the conditions of the method, and the effect of a blood glucose of 100 mg. per 100 ml. would be an apparent blood fructose figure of 2.5 mg. per 100 ml. This effect has been noted by other authors [cf. Radt, 1928; Corley, 1929]. The colour obtained with "fasting" blood appears to be a mixture of a faint trace of blue, due to glucose, with a faint yellow derived from some unknown source. Stewart *et al.* [1937] avoided the difficulty by the use of the photometer, and constructed their calibration curve from a series of fructose solutions made up in filtrates from "fasting" blood.

In all the variations of the diphenylamine method which have hitherto been published, the conditions for the essential reaction between fructose and diphenylamine have been similar. The fructose has been treated with aqueous HCl, an alcoholic solution of diphenylamine has been added, and the mixture has been heated in a boiling water bath. The amount of alcohol included at this stage has been very small—usually 1 part of alcohol to 20 of the aqueous mixture. Under these conditions a considerable amount of the diphenylamine

comes out of solution, and, as the blue compound formed is also sparingly soluble in water, it has been necessary to extract the blue substance with some suitable solvent in order to obtain a clear solution for colorimetric estimation. Workers have used different solvents for this purpose, and have also chosen various methods of preparing protein-free filtrates of blood for analysis, but all have carried out the principal reaction in aqueous solution [Radt, 1928; Corley, 1929; Steinitz & Von Riesen, 1932; Oppel, 1930; Patterson, 1935].

The essential change introduced by the writer is the inclusion of sufficient ethyl alcohol at the stage of colour development to hold in solution both the diphenylamine and the blue compound. Under these conditions the intensity of the colour produced is greatly increased—it is about four times the intensity obtained in Patterson's method. The solution remains clear, and it is therefore unnecessary to perform a subsequent extraction with another solvent. This shortens the procedure and also avoids any possible error due to incomplete extraction. With the increased intensity of colour, it is also possible to reduce the amount of material required for an estimation, and to eliminate the process of concentrating the blood filtrates before analysis. Moreover, the increased intensity of colour simplifies the interpretation of results for blood in the fasting state, as the yellow element in the colour becomes negligible in relation to the blue. The effect of glucose, expressed as fructose, is about the same as in the older methods, but is more easily estimated.

Reagents:

Method

(1) 10 % $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$.

(2) 0.5 *N* NaOH.

(3) Acid-alcohol-diphenylamine reagent: ethyl alcohol, 70 parts; conc. HCl, 50 parts; 20 % diphenylamine in alcohol, 6 parts.

(4) Standard fructose solution: a stock solution of 1 % fructose in saturated benzoic acid is diluted for use. The most convenient standards are:

(a) 0.025 mg. fructose in 2 ml. (equivalent to 5 mg. fructose per 100 ml. plasma).

(b) 0.1 mg. fructose in 2 ml. (equivalent to 20 mg. fructose per 100 ml. plasma).

The titre of the ZnSO_4 and NaOH solutions should be checked as described by Somogyi [1930].

The writer's practice has been to prepare the acid-alcohol-diphenylamine reagent immediately before use, keeping the 20 % alcoholic diphenylamine as a stock solution in a dark bottle, but the mixture of acid, alcohol and diphenylamine keeps well for at least a month; its stability for longer periods has not been tested.

Procedure. Take 2 ml. plasma in a 15 ml. centrifuge tube. Add 2 ml. water and 2 ml. ZnSO_4 solution. Close the tube with a rubber stopper and shake thoroughly. Add 2 ml. NaOH solution and again shake thoroughly. Centrifuge the mixture well and pour the supernatant fluid through a small filter paper (Whatman No. 42, 5.5 cm.). Just over 4 ml. filtrate should be obtained, which suffice for duplicate analyses.

In a pyrex test tube (about $\frac{5}{8} \times 6$ in.) graduated at 10 ml., take 2 ml. filtrate and 6 ml. acid-alcohol-diphenylamine reagent. The standard fructose solutions are similarly treated. Place the tubes in a boiling water bath for exactly 15 min. and then cool them rapidly in running water. Dilute to the 10 ml. mark with alcohol and compare the colours in the colorimeter.

Reasons for the conditions chosen

Time of heating. The colour given by fructose does not reach its maximum intensity until heating has been continued for 25 min. The rate of colour development is most rapid in the earlier stages of heating; 81 % of the maximum colour has been developed after 15 min. and 94 % after 20 min. With glucose the time relations are quite different; the rate is slow at first and becomes much more rapid after 15 min. This is illustrated in Fig. 1, which gives the curves of

Fig. 1. Rates of colour development with glucose and fructose. The colour developed by 0.05 mg. fructose at 25 min. is taken arbitrarily as 100 units. Continuous line: 0.05 mg. fructose ($\equiv 10$ mg. per 100 ml. plasma). Broken lines: I, 0.50 mg. glucose ($\equiv 100$ mg. per 100 ml. plasma); II, 0.75 mg. glucose ($\equiv 150$ mg. per 100 ml. plasma); III, 1.00 mg. glucose ($\equiv 200$ mg. per 100 ml. plasma).

colour development for 0.05 mg. fructose (corresponding to 10 mg. fructose per 100 ml. plasma) and for 0.5, 0.75 and 1.0 mg. glucose (corresponding to 100, 150 and 200 mg. glucose per 100 ml. plasma). It is evident that the effect of glucose, relatively to fructose, is increased when the heating is prolonged beyond 15 min. In Table I the average effect of glucose is given in terms of fructose, on the basis of comparisons with fructose solutions heated for the same time.

Table I. *Apparent fructose equivalents of glucose*

Glucose mg. per 100 ml. plasma	Apparent fructose mg. per 100 ml. plasma		
	After 15 min. heating	After 20 min. heating	After 25 min. heating
100	2.5	3.9	5.8
150	3.7	6.1	8.9
200	4.7	8.0	12.2

As a result of these observations, it was decided to cool the samples after 15 min., in order to keep the glucose effect at a minimum. Since at that time the reaction with glucose is proceeding rapidly, it is necessary to cool the tubes as quickly as possible.

Method of protein precipitation. In Radt's method, the proteins were precipitated by HCl and HgCl_2 . Patterson found that in filtrates prepared by this method the rate of colour development was greater than in the standard solutions

and high results were obtained. He therefore adopted the zinc hydroxide precipitation, but this had the disadvantage that the dilution of the blood could not conveniently be less than 1 in 10. In order to obtain a suitable amount of fructose for analysis it was necessary to concentrate 10 ml. of the filtrate to 4 ml. before the treatment with HCl and diphenylamine. The process of concentrating the filtrates is laborious when large batches of analyses have to be made.

In the writer's method the colour produced by a given amount of fructose is about four times as intense as in Patterson's method. As soon as this was discovered it was evident that an amount of filtrate corresponding to 0.5 ml. blood or plasma could conveniently be used, and the actual colours read would then be twice as intense as in Patterson's method. If blood or plasma is diluted 1 in 4 at the stage of protein precipitation, then 2 ml. filtrate can be conveniently taken for analysis without the necessity of concentrating the filtrate. These volume relations can be maintained when the proteins of plasma are precipitated by zinc hydroxide, and this is the method here adopted.

Some other protein precipitants have been considered and abandoned. With trichloroacetic acid it is easy to maintain the desired volume relations, and the trichloroacetic acid gives no colour with the diphenylamine reagent, but it does increase the rate of colour development with fructose. The use of trichloroacetic filtrates has not been fully explored, but it would evidently be necessary, in applying this method, to include in the standard solution an amount of trichloroacetic acid equivalent to that in the blood filtrates. It would also be necessary to work out afresh the optimum time of heating in order to minimize the effect of glucose.

Experiments have been made with tungstic acid precipitation applied to unclaked blood as in the method of Herbert & Bourne [1930]. It is quite convenient to modify the volume relations in this method so that the blood is diluted 1 in 4, and some satisfactory results have been obtained by this technique. But with some samples turbidities developed during heating with the diphenylamine reagent, and the method is therefore not to be recommended.

For these reasons the zinc hydroxide precipitation has been chosen, and plasma is used rather than whole blood, because with plasma the precipitation can be carried out in a mixture of convenient concentration.

Accuracy of the method

Experiments with pure fructose solutions have shown that the method gives true proportionality of colour to concentration for amounts corresponding to the range from 0 to 40 mg. fructose per 100 ml. plasma.

Table II. *Recovery of fructose added to plasma, mg. per 100 ml.*

Sample ...	1	2	3	4	5	6	7	8	9	10	Average recovery
Apparent fructose of plasma alone	3.0	2.8	2.6	2.4	2.9	3.3	2.9	4.4	2.7	3.3	—
Plasma + 10 mg. fructose per 100 ml.:											
Total found	13.8	12.2	13.4	13.6	13.1	14.4	13.0	15.1	13.6	13.1	—
Fructose recovered	10.8	9.4	10.6	11.2	10.2	11.1	10.1	10.7	10.9	9.8	10.5
Plasma + 20 mg. fructose per 100 ml.:											
Total found	22.1	23.3	22.9	23.7	23.4	—	—	24.2	25.5	23.0	—
Fructose recovered	19.1	20.5	20.3	21.3	20.5	—	—	19.8	22.8	19.7	20.5
Plasma + 40 mg. fructose per 100 ml.:											
Total found	—	—	45.3	—	44.2	—	—	44.8	42.9	43.2	—
Fructose recovered	—	—	42.7	—	41.3	—	—	40.4	40.2	39.9	40.9

The recovery of fructose added to blood was satisfactory (Table II). Of 23 analyses, 17 were correct within ± 1 mg. per 100 ml., and only two showed an error greater than 2 mg. per 100 ml.

The apparent fructose in normal plasma taken in the fasting state ranges from 2.4 to 3.5 mg. per 100 ml. plasma. This is mainly, if not entirely, due to the effect of glucose. The glucose effect is rather irregular, and with a plasma glucose of 100 mg. per 100 ml. one would obtain an apparent fructose value of 2.0–3.2 mg. per 100 ml. (average 2.5). In one experiment with whole blood, a yeast fermentation was carried out by the technique of Van Slyke & Hawkins [1929] on the filtrate after precipitation with zinc hydroxide. The apparent fructose was 3.3 mg. per 100 ml., and all the reacting substance was fermentable by yeast. The effect of the glucose in this sample would be 2.2 mg. per 100 ml. expressed as fructose. The difference, 1.1 mg., may have represented true fructose or may have been due to the irregularity of the glucose effect. It is evident that there is very little, if any, fructose in normal fasting blood.

Whenever absolute values for blood fructose are required, it will be necessary to estimate the total blood sugar and make the necessary correction for the effect of glucose. But for the purpose of the fructose tolerance test in non-diabetic subjects it will not be necessary to estimate the glucose. The rise in blood fructose after the ingestion of fructose can be estimated as an increment above the apparent initial level. It is true that fluctuations in blood glucose occur in the course of the test, but the variations in non-diabetic subjects are too small to cause a significant difference in the apparent fructose figure, and it is therefore justifiable to treat the glucose effect as constant throughout the experiment. In diabetics, the blood glucose shows a considerable rise after the ingestion of fructose, and in these cases it is desirable to estimate the total sugar and calculate the glucose effect.

With this new technique the estimation of fructose can be carried out quite as quickly as the estimation of total sugar, and the method of estimating fructose rather than total sugar in the fructose tolerance test can be adopted in clinical laboratories without the expenditure of any additional time and labour.

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