ON SOME APPLICATIONS OF SAFRANIN AS A TEST FOR CARBOHYDRATES

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Safranin was first suggested as a test for sugar by Crismer¹, who
1. Pharm. Zeit., Vol. XXXIII, p. 65; Pharm. Journal [3], Vol. XIX, p. 348.
 recommended it as a suitable reagent for the detection of glucose in urine. Since that time the reagent has been employed by various observers, but no satisfactory conclusion with regard to the exact usefulness of the test from the clinical standpoint has been forthcoming. For certain purposes, however, as will be shown below, it seems to hold a very definite position, while, on the other hand, its general usefulness can only be appreciated after certain disadvantages are understood and allowed for.

Safranin occurs in commerce as a reddish or brownish-red powder, readily soluble in water to form a blood-red solution which does not readily undergo decomposition on keeping. The addition of caustic alkali causes no immediate precipitate if the alkali is not too concentrated, but there is a tendency for the formation of a precipitate on standing; the stronger the alkali, the more readily a precipitate separates out. If a solution of glucose be added to the alkaline solution and the mixture heated to the boiling point, the red colour is discharged, and the liquid changes to a pale straw-yellow colour; in the presence of a good deal of sugar it becomes turbid from the separation of the insoluble leuco-derivative. In the presence of small amounts of sugar the red colour returns on agitating the liquid or exposing it to the air.

Commercial safranin seems to vary a good deal in composition, but according to Allen¹ is a mixture of several homologous bodies, of

1. Chemistry of Urine, 1895, p. 83.

which $C_{19}H_{17}N_4Cl$, $C_{20}H_{19}N_4Cl$, and $C_{21}H_{21}N_4Cl$ are the chief. The second of these has the constitution of a chloride of paramido-phenyl-paramido-phenazonium, and is said to have the following structural formula :—

Unlike many other tests for sugar, safranin is absolutely unaffected by uric acid or urates, hippuric acid, creatinin, creatin, chloral, chloroform, hydrogen peroxide, salts of hydroxalamine, pyrocatechin or hydroquinone ; in short, the reaction seems to be entirely limited to substances of a carbohydrate nature. The following carbohydrate materials have been tested, the results being indicated by a plus sign where reduction was obtained, and by a minus sign where no reaction was given.

POLYSACCHARIDS

Starch — Ordinary samples may give a slight reaction but purification indicates that the reduction is due to impurities.

Dextrin + Sample used was obtained from Merck ; after precipitating several times with alcohol a well-marked reaction was still obtained.

Glycogen + Not very well marked reaction.

DISACCHARIDS

Cane sugar +

Maltose +

Lactose +

Iso-maltose + (?)

MONOSACCHARIDS

Dextrose +

Laevulose +

Galactose +

PENTOSES AND OTHERS

Xylose +

Arabinose +

Raffinose — (?) very slight reaction, probably due to impurities.

Glycuronic acid + gives very marked reaction.

Mucin —

Mucin boiled with HCl = glucosamine hydrochloride ; gives distinct reaction.

The above results indicate that safranin is essentially a reagent for indicating the presence of carbohydrate in general, and is of itself quite unsuitable for determining the presence of glucose in a mixture where other carbohydrates may be present. Every urine gives a fairly distinct reaction with safranin, and Crismer interpreted this as proving the presence of sugar in normal urine; if 'sugar' be used in the sense of carbohydrate matter in general, this seems to be true, but the nature of the carbohydrate is not directly indicated. The most marked use of the substance seems to be for the detection of small traces of carbohydrate matter, and for this purpose it seems to be the best and most easily applied reagent. The only important substance outside the carbohydrate group that interferes with the reaction is albumin. It is stated by Allen that albumin, while decolorising safranin but very slowly, yet, in the end, caused complete discharge of the red colour. This statement has been generally accepted and is still held by recent observers.

Albumin, however, has but little effect in causing a decolorisation of safranin, and the statement that long boiling causes complete discharge of the colour seems to be quite unfounded. If egg albumin is used, there is, of course, a certain amount of carbohydrate present as well, which no doubt is mainly responsible for any slight reduction apparently caused by the protein. Pure albumin appears to have no appreciable reducing action, and prolonged boiling with protein matter alone never results in the complete discharge of the red colour. When a fluid containing albumin is mixed with the alkaline safranin solution the mixture assumes a pinkish tint, as against the ordinary reddish colour, and this seems to be the most characteristic action of protein material on safranin. Solutions of alkaline safranin with albumin, after boiling for from thirty to sixty minutes, did not discharge the colour from even weak solutions of the reagent, and it would seem that the alleged direct reducing action of albumin, if it exists at all, is so insignificant as to render it of no importance, even in the case of comparatively weak safranin solutions.

For the detection of small amounts of sugar, however, albumin must be removed, for it tends to inhibit somewhat the reducing power

of the sugar on safranin ; a solution of glucose which completely discharges the colour from a certain amount of safranin will not give nearly so marked a reaction if some albumin be previously added to the mixture. In this case it will decolorise less safranin than in a protein-free solution, and for quantitative examination especially it is of importance to remove any protein that may be present in the solution before adding the safranin.

Another substance which somewhat interferes with the reaction is ammonia ; if the latter is present in excess it acts in much the same manner as albumin and prevents the sugar from discharging the red colour as completely as it would otherwise do.

THE REACTION OF NORMAL URINE WITH SAFRANIN

Every urine gives a more or less definite reaction with safranin as a result of the carbohydrate material present in normal urine. In order that the test should prove of practical utility for the detection of pathological amounts of sugar in urine, it is necessary to make allowance for this normal reaction ; and a lack of proper appreciation of the intensity of this reaction given by urine from healthy persons has undoubtedly helped to bring discredit on the test and to give rise to much confusion. By means of safranin very accurate determinations of the amount of carbohydrate material present in normal urine can be made with comparatively small amounts of urine. For a rough quantitative examination of the amount of reducing substances present in a sample of urine it is customary to use a 0.1 per cent. solution of safranin in water ; 1 c.c. of this solution is added to 1 c.c. of sodium hydroxide (5 per cent.) and the mixture is shaken up with 1 c.c. of the urine to be tested, and heated to boiling point ; if sugar is present in sufficient amount the red colour is discharged and the solution becomes yellow. In performing the test care should be taken to agitate the liquid as little as possible in order to prevent reoxidation of the reduced leuco-derivative. The general statement that 1 c.c. of a 0.1 per cent. solution of safranin is reduced by 1 c.c. of a 0.1 per cent. solution of chemically pure glucose is really inaccurate. In several samples of pure glucose obtained from Merck and from

Grübler it was found that from 1.5 c.c. to 2 c.c. of 0.1 per cent. solution of safranin was decolorised by 1 c.c. of a 0.1 per cent. solution of glucose ; different samples of safranin were used. In short, 1 c.c. of glucose solution will decolorise more nearly 2 c.c. of the same strength of safranin than 1 c.c., as generally stated. In the quantitative estimation of sugar it is quite impossible to obtain even an approximately quantitative indication of the amount present by adding safranin to the hot alkaline sugar solution as long as it is decolorised and subsequently calculating the amount of safranin solution used in terms of glucose. This simple plan has been advocated, but so many disturbing factors enter into the calculation as to render the results very inaccurate, and by no means constant for the same solution. Material differences are obtained as a result of the different periods of time taken to add the necessary amounts of safranin to the sugar solution ; for a considerable quantity of the sugar is destroyed by the hot caustic alkali and, naturally, the greater the interval between each addition of safranin the lower the total amount of sugar present appears to be. Again, the gradual addition of safranin causes agitation of the fluid, and this increases the tendency to reoxidation to such an extent as to very markedly interfere with the result. Further, only the roughest indication of the sugar actually present can be obtained by the above method. In short, safranin is not a satisfactory reagent for quantitative examination of fluids containing a large amount of sugar, since quicker and more accurate results can be obtained by other means ; on the other hand, it gives a rough general indication. For the estimation of such small amounts of sugar as cannot be satisfactorily ascertained by other tests, safranin, when carefully used, is, in the writer's opinion, one of the most useful of reagents, being both accurate and easy of application when special precautions are adopted ; its delicacy renders it of great value in ascertaining slight changes in the total amount of carbohydrate present in any fluid as the result of the reaction of a reagent or ferment.

By means of this test the presence of a distinct quantity of fermentable sugar (which may be taken as glucose) can be easily indicated in every urine, and this method affords an easy means of demonstrating

the presence of glucose in normal urine to a class of students. The quantity of fermentable sugar present in ordinary urine is so small as to render the results of fermentation exceedingly doubtful; any CO_2 that is evolved is so slight in amount as to be easily held in solution by the fluid; again, if there is any evidence of fermentation having taken place, there is practically no clue obtained as to the amount of sugar actually present in terms of the CO_2 evolved.

With safranin, however, the exact amount of fermentable sugar present can be easily determined, and the following simple plan for showing the presence of sugar in urine is quite striking in regard to the constancy of the results obtained.

About 50 c.c. urine is well mixed with some bakers' yeast and placed in the incubator at 40°C . for from eighteen to twenty-four hours. In order to keep the yeast thoroughly mixed with the solution it is shaken up at intervals. After fermentation has taken place the yeast is filtered off, and the necessary corrections made to allow for evaporation. A comparison of the fermented urine with a specimen of the same urine unfermented at once reveals the fact that the reducing power of the fermented specimen is now much less than that of the unfermented part. For example, a urine of which 1 c.c. completely decolorised about twenty-four drops of a 0.1 per cent. safranin solution before fermentation, was changed by fermentation to such an extent that under similar conditions 1 c.c. now decolorised only about eleven drops.

Besides reducing fermentable sugar, urine contains a certain proportion of unfermentable carbohydrate, for in no instance has it been possible to obtain a urine which gave no reaction with safranin after fermentation; in fact, the unfermentable residue is generally sufficient to give a fairly pronounced reaction. This seemed a good and easy method for determining the relative quantities of fermentable and non-fermentable carbohydrate-like substances in urine, but the question whether the apparently unfermentable substance might not be really fermentable sugar which had escaped fermentation owing to the inhibiting action of some of the normal constituents of urine had first to be answered. Again, it was possible that the products of

yeast ferment activity might render it impossible to eliminate the last traces of fermentable sugar in even a watery solution.

In order to test this, weak solutions of sugar (from 0.05 per cent. to 0.2 per cent.) were made up in distilled water and fermented with yeast in an open flask placed in the incubator at 40° C. Similar solutions containing as nearly as possible the correct percentage of the chief constituents of normal urine were made up and tested in the same way. In every case it was found that for practical purposes the sugar was entirely destroyed, or if there was any reaction it was so slight as to be of little importance.

The following results were obtained with above solutions, 1 c.c. of the fermented fluid being mixed with 1 c.c. sodium hydrate solution: the number of drops of safranin solution decolorised shows the extent to which fermentation had gone on.

Solution				No. of hours fermented	No. of drops of 0.07% safranin solution decolorised after fermentation
Glucose	0.05 %	in dist. H ₂ O		18	1.5
„	0.1 %	„		23	1
„	0.15 %	„		21	1.5
„	0.05 %	+ urea		24	2
„	0.1 %	+ „		22	2
„	0.12 %	+ uric acid (alkaline fluid)	...	19	2
				18	2
„	0.12 %	+ chlorides		18	2.5
„	0.12 %	+ phosphates		22	2
„	0.12 %	+ sulphates		21	2.5
„	0.15 %	+ mixture of ordinary urine constituents		20	2

From the above it is obvious that none of the ordinary constituents of urine have any appreciable effect in inhibiting the action of yeast or in preventing its activity in the presence of very small amounts of sugar.

The effect of small amounts of acid and alkali was also investigated. The results obtained seemed to indicate that fermentation was accelerated to some slight extent by the presence of a very faint trace

of alkali in the sugar solution. On the other hand, small amounts of acid or alkali, sufficient to give a very distinct litmus reaction to the fluid, did not inhibit the reaction to any appreciable extent. With strong acids and alkalies my results were in agreement with those obtained by Drabble and Scott¹—fermentation was either retarded or completely prevented. In testing the above safranin was exceedingly useful, for it is obvious that in alkaline fluids no importance can be attached to the absence of CO₂, the gas, if evolved, being rapidly absorbed by the alkaline medium.

Again, some experiments were performed in which the urine was evaporated to a half, a third, and a quarter its bulk. If the residual substance was really an unfermentable carbohydrate the residue after fermentation should bear a constant and inverse ratio to the artificial concentration of the urine—a urine evaporated to say one-third of its bulk should, after fermentation, show three times as much residual substances as the same urine before concentration. This was always found to be the case, so that the substance remaining after fermentation seems really to be an unfermentable carbohydrate body.

ON THE TOTAL AMOUNT OF CARBOHYDRATE IN NORMAL
URINE AND THE RELATIONSHIP OF THE UNFERMENTABLE
TO THE FERMENTABLE PART

Certain experiments were performed with safranin in order to determine accurately the total amount of carbohydrate present in normal urine, calculated as glucose. A series of solutions of chemically pure glucose were made up in strengths of '02 per cent., '03 per cent., etc., up to '2 per cent., 1 c.c. of each of these solutions was mixed with 1 c.c. 5 per cent. NaOH and to this was added such an amount of safranin as was readily decolorised by the amount of sugar present after the mixture was heated for some time. In order to insure the equal distribution of heat to two or more tubes at the same time, they were placed in a beaker of water just kept at boiling temperature. Three solutions of safranin were used—one a '1 per cent.

1. *Bio-Chemical Journal*, Vol. II, p. 340.

solution and the others '15 per cent. and '2 per cent., and the number of drops of a suitable solution that was decolorised after heating for about half a minute was determined, and the glucose solution labelled accordingly. Equal quantities of urines were treated against these artificial solutions; thus the strength of glucose solution which exactly corresponded in its intensity of decolorisation and the time taken for the reaction with any given urine indicated the reducing power of that particular sample in terms of glucose.

When the urine did not quite correspond in reaction to any of the glucose solutions, the result midway between two solutions was taken; for instance a urine which gave a reaction a little more marked than a '11 per cent. solution of glucose and yet not quite so marked as a '12 per cent. solution, was taken as indicating the presence of carbohydrate matter equivalent to '115 per cent. glucose.

The following table indicates the results obtained in twenty normal urines.

No.	Specific gravity	Before yeast (Total carbohydrate present)	After yeast (Unfermentable carbohydrate present)	Fermentable substance present
1.	1020	0'11 per cent.	0'055 per cent.	'055 per cent.
2.	1021	0'14 „	0'06 „	'08 „
3.	1015	0'10 „	0'04 „	'06 „
4.	1022	0'125 „	0'06 „	'065 „
5.	1025	0'11 „	0'05 „	'06 „
6.	1026	0'115 „	0'045 „	'07 „
7.	1025	0'10 „	0'035 „	'065 „
8.	1020	0'095 „	0'035 „	'06 „
9.	1014	0'08 „	0'035 „	'045 „
10.	1015	0'085 „	0'03 „	'055 „
11.	1023	0'10 „	0'045 „	'055 „
12.	1021	0'125 „	0'04 „	'085 „
13.	1015	0'085 „	0'03 „	'055 „
14.	1016	0'09 „	0'04 „	'05 „
15.	1018	0'11 „	0'06 „	'05 „
16.	1022	0'08 „	0'025 „	'055 „
17.	1025	0'135 „	0'045 „	'09 „
18.	1024	0'14 „	0'06 „	'08 „
19.	1024	0'10 „	0'05 „	'05 „
20.	1023	0'095 „	0'035 „	'06 „

Several hundred normal urines were examined, and it was found, as a general rule, that urine of average specific gravity from a healthy person contains on an average carbohydrate matter equivalent to from about '08 per cent to '1 per cent. glucose. A glance at the table indicates that the ratio of unfermentable to fermentable carbohydrate present is fairly constant, and varies roughly from 1 : 3 up to 1 : 2. In other words, from one-third to one-half of the total carbohydrate material in the urine is unfermentable carbohydrate. In many specimens the amount of fermentable sugar is in considerable excess of the unfermentable, though the limit is by no means great.

A normal urine, therefore, contains roughly carbohydrate to the equivalent of about 1 gramme to 1·5 grammes glucose per diem ; of this, from '33 gramme to '75 gramme is unfermentable carbohydrate material, probably largely iso-maltose, while the rest is probably glucose.

That glycuronic acid is not of much importance is indicated by the fact that removal of any traces of this compound does not seem to materially affect the relative proportions of the two groups ; this point, however, I intend to work out later on.

Several urines obtained from patients suffering from various diseases were examined from time to time. As a general rule it was found that the total carbohydrate varied but little, while the ratio of fermentable to unfermentable substance was generally much the same as in normal urine.

The following are the result of a few urines obtained from diseased patients.

No.	Specific gravity	Nature of case	Before fermentation (Total reducing carbohydrates)	After fermentation Total unfermentable carbohydrate	Total fermentable carbohydrate
1.	1028	Malignant disease of liver and colon	'115 per cent.	'05 per cent.	'065 per cent.
2.	1018	Pancreatic disease	'06 „	'025 „	'035 „
3.	1025	Malignant disease of the pylorus	'10 „	'055 „	'45 „
4.	1042	Diabetes... ..	6'5 „	'035 „	6'465 „
5.	1020	Chronic Bright's disease	'10 „	'035 „	'065 „
6.	1018	Pernicious anaemia	'090 „	'035 „	'055 „

Assuming, therefore, that the fermentable substance of ordinary urine is glucose, it would appear that ordinary urine contains roughly from '03 per cent. to '07 per cent. glucose. Such an amount agrees with the results obtained in testing by Fehling's solution for the creatinin present can easily prevent any reaction. In testing a urine that gives an ambiguous sugar reaction with other tests, safranin is exceedingly useful owing to the fact that ordinary 'interfering' substances, such as uric acid and creatinin, do not affect it. As a general rule it may be assumed that a urine which completely decolorises an equal quantity of a '25 per cent. to '3 per cent. safranin solution contains sugar in abnormal amount; in order to make certain whether the substance is glucose the urine ought to be fermented with yeast in an open flask at suitable temperature for twenty-four hours or so; after filtration, the contents of the flask should be again tested with safranin, and the diminution in reducing power noted. This excludes all possibility of interference by glycuronic acid and other substances, and gives an indication of the amount of sugar actually present. At the same time it is useful in a doubtful case in ascertaining whether the ambiguous reaction was due to pathological excess of sugar or merely physiological as the result of concentration. If the former is the case the ratio of fermentable to unfermentable substance may be high, say from 6 or 8 : 1 or even more; in the latter case the ratio will remain as usual—from 3 or 4 : 1 up to 2 : 1.

In all cases where urines give a doubtful reaction for sugar with such tests as Fehling's solution the writer has found the above plan very satisfactory and easy of application; the only disadvantage is that it takes at least a day, but at present there does not seem to be any very reliable and quick test for the detection of small amounts of sugar.

OTHER USES OF SAFRANIN

Safranin is therefore an excellent reagent for determining slight differences in the case of small amounts of sugar present in different solutions, or in the same solution at different times. In digestion

experiments the amount of starch changed can be comparatively easily obtained, while the differences in the carbohydrate contents of urine or blood, after standing for some time, as the result of glycolytic action, can be easily and fairly accurately determined. So far as my experiments go I have been unable to detect any appreciable reduction in the amount of sugar in urine after standing for some days, provided micro-organisms be excluded; it is intended, however, to work out these points with regard to blood and urine more fully later on. The above serves to indicate the uses of safranin for solutions containing small amounts of carbohydrate, and while the reagent possesses certain disadvantages with regard to its use as a routine test for the examination of urine in unskilled hands, it often proves exceedingly useful for the detection of traces, especially when combined with yeast fermentation.

CONCLUSIONS

1. Safranin is a general test for carbohydrate bodies of a certain type, and is one of the most suitable reagents for determining the presence of traces of carbohydrates in liquids; it is unaffected by all the ordinary 'interfering' substances of urine except those of a carbohydrate nature.

2. Safranin is not decolorised by albumin after long boiling, but the presence of albumin in the liquid to be tested interferes with the delicacy of the reaction; ammonia in excess acts in a somewhat similar manner.

3. By a combination of safranin and fermentation by yeast fermentable sugar can be easily demonstrated in every urine; along with the fermentable sugar, which is probably glucose, there is always present a definite amount of unfermentable carbohydrate.

The ratio of fermentable to unfermentable substances in normal urine varies roughly from 1 : 3 to 1 : 2; if the ratio is much disturbed the urine is probably pathological.