CXLI. THE USE OF SOME MICRO-ORGANISMS IN SUGAR ANALYSIS.

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BACTERIOLOGISTS have long used gas or acid production from a series of sugars for the identification of bacteria. Conversely, there are systems, or partial systems, of identification of sugars based on the fermentative action of different organisms [Castellani and Taylor, 1917; 1919; 1922; Kendall, 1923; Kendall and Yoshida, 1923].

From a qualitative standpoint such reactions may have been satisfactory; quantitatively, their application has not been always successful. Early criticisms of these methods have been made by MacLeod [1921] and Høst and Hatlehol [1920].

Two improvements have, however, rendered sugar estimation by living organisms less subject to error. Hiller, Linder and Van Slyke [1925] used a large amount (approximately 250 mg.) of baker's yeast to ferment the sugar in 1 cc. of blood, the optimum time of incubation necessary to produce the greatest diminution in reducing power being 20 mins. at 38°. Somogyi [1927] washed the yeast until free from reducing substances, prior to its use. Since then there have been many variations in the determination of "fermentable sugar" by yeast, but all depend on the employment of a large amount of washed yeast, a small amount of sugar and a short period of incubation. It is possible, however, that we may require sharper definitions of the volumes and nature of the interacting cells and fluid. Thus Schrumpf [1932] denies the possibility of the separation of glucose and galactose by yeast, and suggests that the non-fermentable portion of "blood-sugar" is a secondary product of the yeast on the glucose.

Raymond and Blanco [1928, 1] found that fructose, mannose and sucrose are partially removed from solution by baker's yeast under conditions favourable to the complete removal of glucose, whilst galactose, arabinose, xylose and dihydroxyacetone are untouched. Harding and Grant [1931] showed that if baker's yeast is acclimatized to ferment galactose, it can then be used to remove galactose from solution under conditions analogous to the estimation of glucose by the original yeast. *Saccharomyces marxianus* [Harding, Nicholson and Grant, 1932-33] which also ferments galactose, has been found useful as an analytical reagent for the same sugar; moreover, this yeast does not possess the power either to ferment or remove maltose.

These results make clear the existence of a connection between the fermentative action of a yeast, and the ability of the same yeast to act as an analytical reagent under specified conditions. The present paper shows that the principle is capable of further extension, and it seems within the bounds of possibility that a complete system of analysis of carbohydrate mixtures may ultimately

be devised. We have been able to analyse a mixture of glucose, fructose, galactose, maltose, lactose and sucrose within an error of 7 %, using the limited number of biological reagents as yet available. Such methods of analysis are particularly valuable when only small amounts of sugar are available.

In attempting to extend the use of biological reagents to the quantitative removal of sugars from dilute solutions in this manner, we have turned naturally to those yeasts and bacteria which have already proved useful in the qualitative detection of sugars. We thus have examined a series of *Monilia* species (kindly supplied to us by Sir Aldo Castellani) and a number of strains of *Proteus vulgaris*. From these we have selected a few which seem valuable from our point of view.

The use of pure strains of organisms becomes a necessity if precise and reproducible results are to be obtained. The experience of Harding and Grant with galactose-acclimatised baker's yeast, which shows a variable action towards maltose, and the even more variable action towards maltose of Fleischmann's baker's yeast, as shown in this paper, illustrates this necessity.

EXPERIMENTAL METHODS.

Sugars were bought as pure and twice recrystallised. Approximately 0.1 % aqueous stock solutions were made, and from these were prepared the various mixtures and dilute solutions used in the experiments. The reducing values before and after the action of the yeast under examination were determined on 2.0 cc. of the sugar solution + 2.0 cc. of a modified Shaffer-Somogyi copper reagent, heated for 10 mins. in a rapidly boiling water-bath [Harding and Selby, 1931; Harding and Downs, 1933]. As far as we know any sensitive copper or ferricyanide solution can be used.

The cultural conditions of the *Proteus vulgaris* and the *Monilia* species are given under separate headings. Those of *Saccharomyces marxianus* have already been published [Harding, Nicholson and Grant, 1932-33].

Fermentation and removal power.

To signify the loss in reducing power suffered by a sugar solution when treated with a large excess of a yeast for a short period of time, we have in this and previous papers used the term "removal." Hiller, Linder and Van Slyke used the term "fermentation." Benedict [1928] also selected this term, though so far no evidence has been presented that under the particular conditions of the reaction the processes usually designated as alcoholic or acid fermentation are actually responsible for the removal of the sugar. Raymond and Blanco [1928, 1] used the terms "adsorption" and [1928, 2] "disappearance," the latter term denoting our lack of precise knowledge of this phase of yeast action. The closeness of the connection, however, between fermentative ability and removal power can be seen by a comparison of the removal power of the organisms on a series of sugars, shown in Table I, and the gas or acid production as shown in Footnote to Table I.

The main lack of correlation between fermentation and removal power is with galactose. There is no gas or acid production by *M. pinoyi*, yet 100 % removal is effected. *P. vulgaris* is inert fermentatively, yet galactose suffers a small removal loss. *M. krusei*, which produces neither gas nor acid from galactose, and does not remove any galactose from solution when the sugar concentration is 20 mg./100 cc. or less, removes a small percentage when the sugar concentration is 50 mg./100 cc. This is not true of the action of *M. krusei* on maltose, lactose or arabinose. Both *S. marxianus* and *M. macedoninensis*

Table I. *Showing percentage removal by some organisms of a series of sugars.*

Organism	Washed wet-weight g.	Incubation time mins.	Glucose	Fructose	Mannose	Galactose	Maltose	Lactose	Arabinose	Xylose	Sucrose	mg. sugar in 10 cc. solution
<i>Proteus vulgaris</i>	0.75	30	100	0	0	21	0	0	0	0	0	1.5
<i>Monilia krusei</i>	0.5	30	100	99	100	0	0	0	0	—	0	2
<i>Saccharomyces marxianus</i>	0.5	30	100	100	100	100	0	0	0	0	100	2
<i>Monilia tropicalis</i>	0.5	20	100	100	100	28	100	0	0	0	5	2
<i>Monilia pinoyi</i>	0.5	30	100	93	90	69	100	0	0	—	0	2
"	0.75	30	100	99	100	80	100	0	0	—	0	2
"	1.0	30	100	100	100	100	100	0	0	—	0	2
<i>Monilia macedoninensis</i>	0.5	30	100	100	100	21	0	0	0	—	100	2

The removals have been carried out in neutral aqueous solution.

Footnote to Table I.

The gas or acid production was determined on a sterile 1 % solution of the pure sugar in peptone broth by the usual bacteriological technique.

Proteus vulgaris. Glucose: acid 12 hours, gas 72 hours. Fructose, mannose, galactose, maltose, lactose, arabinose, xylose, sucrose: no acid or gas in 72 hours.

M. krusei. Glucose, fructose, mannose: acid and gas 12 hours. Galactose, maltose, lactose, arabinose, xylose, sucrose: no acid or gas in 72 hours.

S. marxianus. Glucose, fructose, mannose, galactose, sucrose: acid and gas 12 hours. Maltose: acid in 48 hours, no gas in 72 hours. Lactose, arabinose, xylose: no acid or gas in 72 hours.

M. tropicalis. Glucose, fructose, mannose, maltose: acid and gas 12 hours. Sucrose: acid 12 hours, gas 72 hours. Galactose, lactose, arabinose, xylose: no acid or gas in 72 hours.

M. pinoyi. Glucose, fructose, mannose: acid and gas 12 hours. Maltose: acid 12 hours, gas 48 hours. Galactose, arabinose, xylose, sucrose: no acid or gas in 72 hours.

M. macedoninensis. Glucose, fructose, mannose, sucrose: acid and gas in 12 hours. Maltose: acid in 12 hours, no gas in 72 hours. Galactose, lactose, arabinose, xylose: no acid or gas in 72 hours.

slowly produce acid from maltose, but no gas in 72 hours, and neither shows removal power under our conditions. An alteration in properties is also to be noted in another section of the present paper where ordinary baker's yeast is found to have a removal action on maltose in a concentration of 20 mg./100 cc. but not at 10 mg./100 cc.

With the exceptions just noted, the removal power of a yeast, however, appears to be a function parallel with its rapid fermentative ability. Yet we hesitate to identify completely the two properties, quite apart from the fact that the galactose removals can be taken as evidence of the existence of an action other than fermentation. The removal of glucose can be extraordinarily rapid, so rapid indeed, that Somogyi [1927] at first considered the reaction as instantaneous. In all our experiments, 8 mins. has been sufficient to ensure the removal of 2–5 mg. of glucose by 250 mg. yeast at 38°. Fructose and mannose, which generally go through the same fermentative processes, require a much longer time to effect their complete removal, though by far the greater part of the removal is effected in the first few minutes. In this connection we would draw attention to the finding of Slater and Sand [1910] that the rate of entrance of glucose into the yeast cell is always more than sufficient (in presence of excess sugar) to maintain the maximum rate of fermentation. It seems to us a possibility that the sugar removal as shown under our analytical conditions may be associated with this rapid entrance into the yeast cell preliminary to the true fermentation process.

It is important to draw attention to the sharpness with which sugars can be separated by these methods. Not only is there a complete removal of the fermentable sugar, but there is no diffusion of the non-fermentable sugar in the yeast layer. Somogyi [1927] first noticed this, and it is illustrated by many

experimental figures in this paper. Van Slyke and Hawkins [1929] put this part of the technique severely to the test, when they incubated a 1 : 5 Folin-Wu blood-filtrate (previously treated with a small amount of yeast to remove the glucose) with an equal volume of centrifuged washed yeast, and found no alteration in the concentration of the residual non-fermentable substances. This, they found was not true of urine, though Harding and Selby [1931] and West and Peterson [1932] have been unable to confirm the removal of non-fermentable substances from urine under their own conditions.

The use of Proteus vulgaris as a reagent for glucose.

From the results of Table I it appears that *Proteus vulgaris* can be a valuable reagent for glucose. Our strain possesses neither fermentative ability nor removal power towards fructose, mannose and sucrose, and it would be in this field that it would prove most useful. The separation of glucose from sugars other than these, can be effected by many yeasts which are much more easily handled than this bacterium. However, the importance of glucose in the mammalian organism, and the value of a method for quantitatively separating this sugar from fructose and mannose, warrant the use of this bacterium as a sugar reagent, difficult as we have found the practical details of its application. Castellani and Taylor [1917; 1919; 1922] had previously reported *Monilia balcanica* as a specific gas-producer from glucose, but this organism is no longer available.

Strain. Of the three strains available from the American Type Culture Collection and three strains from the Department of Bacteriology of this University only one of the latter proved suitable. This particular strain was isolated 7 years ago by Prof. Holman. It has retained the same sugar-removal properties during the 2 years' work in this Department.

Culture. The bacteria are grown on 2 % neutral glucose-peptone broth for 24 hours at 38.0°. One loopful is transferred to a corner of 2 % neutral glucose-agar surface contained in a 26 oz. flat narrow-necked bottle and incubated for 24–36 hours at 38.0°. The growth is characteristic, spreading very rapidly from the inoculation corner over the whole agar surface. This habit of vigorous surface spread has assisted greatly in keeping a pure strain. The growth is washed off with 10 cc. distilled water for each bottle¹.

Separation. The bacteria can be separated from 1–2 cc. of liquid by the ordinary centrifuge at 3500–4000 r.p.m., but, with larger amounts of fluid there is always a considerable suspension, even after prolonged centrifuging. Pressure filtration through Seitz bacterial filter-pads, through Berkefeld or cellophane filters has proved too slow. The use of the Seitz pads, in addition gave products containing large amounts of reducing substances. The angle centrifuge of Lundgren at 3500 r.p.m. separated the bacterial suspension in 5 mins. Neither the ovoid glass centrifuge-tubes supplied by the manufacturer nor similar tubes made of pyrex glass proved satisfactory. Finally, thick aluminium tubes, made locally, were found serviceable. The lack of visibility inside these tubes was not found to be a hindrance.

Washing and yield. After the first centrifuging, the packed bacteria are stirred with 10 cc. distilled water and re-centrifuged. This is repeated 4 or 5 times. Usually the bacteria are then free from reducing substances. This method of washing is essential if, at the end of the action on the sugar solution, the bacteria are to be removed by centrifuging only. If, however, this latter removal is to be accomplished by filtration through kieselguhr it is sufficient to carry out only the first separation and one subsequent washing. From 1.0 to 1.5 g. of washed packed bacteria are obtained from each 26 oz. bottle.

Stability. The washed bacteria in 50 % suspension in water have shown variable stability. Some preparations kept at 4° have remained constant in their glucose-removal power for 4 days.

¹ The organism is kept in the same tube of broth culture for one week. Each week a loopful is transferred to an agar plate and re-transferred to broth after 24 hours. A stock culture is kept on an agar slant in a sealed test-tube and is transferred from agar to broth and back to agar once a month.

Other samples in the same length of time have altered, not only in glucose-removal power but in specificity. We have used the organisms within 12 hours of preparation.

Estimation of glucose. 1.5 cc. of 50 % suspension of the *Proteus* preparation are centrifuged in the aluminium tubes, the supernatant liquid poured off and adherent moisture removed from the tube by filter-paper. 10 cc. of the neutral sugar solution to be tested, containing not more than 1.5 mg. glucose are added, and the mixture stirred with a thin glass rod at 38° for 30 mins. The solution should now be at p_H 6.0–6.5 due to acid formation. 0.1 g. solid KH_2PO_4 is added if this is not attained. (In acid solution the bacteria flocculate rapidly and are more readily removed by centrifuging.) The greater part of the bacteria is then removed by a short period in the angle centrifuge. The removal of the last few cells must be accomplished separately by one of three methods. (a) By use of the thin high efficiency centrifuge-tubes supplied with the high angle centrifuge by the manufacturer. These tubes, if protected by rubber coverings, are satisfactory. The tubes hold only 5–7 cc. however, and in consequence a considerable time may be spent in this second centrifuging, if some 40–60 cc. liquid must be handled. (b) By filtration through a Berkefeld candle. This takes about 2 hours for 40–60 cc. liquid, but requires no attention. (c) By filtration through a layer of coarse kieselguhr on a small Büchner filter-plate. The kieselguhr is previously washed with HCl. Filtration is rapid, and if a considerable volume of liquid is available, this offers the best method for the removal of bacteria. The filtrate is bacteria-free, as tested by cultures, and sugars pass quantitatively. It is not possible to dispense with the first centrifuging, for if too many bacteria are present, the layer of kieselguhr clogs.

If the centrifugate or filtrate from (a), (b) and (c) contains ammonium salts, they are removed at this stage [Harding and Downs, 1933]. The reducing value of the sugar solution before and after *Proteus* action¹ is determined, using the requisite water controls. The difference between total and residual sugar represents glucose, in absence of galactose.

Action on galactose. The maximum removal of galactose we have observed is 27 % of a 20 mg./100 cc. solution, using 0.75 g. bacteria on 10 cc. of the neutral aqueous sugar solution for 30 mins. Using the same volume of sugar solution, bacteria and time of incubation, as under the previous subsection, we can estimate glucose in presence of galactose, following the summary of directions in the final section of this paper.

Action on urea. Different strains of *Proteus vulgaris* have been found to possess hydrolytic action on urea, producing ammonium carbonate [Day *et al.*, 1930]. 0.75 g. of wet-weight washed bacteria of our strain hydrolyses 10 cc. of a 250 mg./100 cc. urea solution to approximately 50 %. Such amounts of ammonia have been shown to lead to low results in sugar estimations [Harding and Downs, 1933]. As the conditions of the urea solution simulate a 1 in 10 diluted urine it becomes imperative, either to remove the urea, or to remove the NH_3 formed by its decomposition, before attempting sugar determinations by means of *Proteus*.

Recently Larson [1932] has discussed the problem of urea and NH_3 removal in presence of sugars without arriving at a satisfactory conclusion. We examined the removal of urea from urines by the use of $HgSO_4$ followed by either Na_2CO_3 or $BaCO_3$ [West and Peterson, 1932]. Even a second treatment with the mercuric salt still left sufficient urea to act as a source of NH_3 . The use of urease followed by permutit required such large quantities of reagents as to render the process impracticable for our method of sugar determination. Permutit, after the action of *Proteus*, gave high sugar recoveries. The removal of NH_3 as $Mg(NH_4)PO_4$ by the addition of KH_2PO_4 followed by MgO was found satisfactory after *Proteus* [Harding and Downs, 1933].

Analysis of glucose, fructose and sucrose. This mixture of sugars is important, both commercially and biologically. Given sufficient material the determination of the reducing power, before and after hydrolysis, together with the rotation of the mixtures provide data for the analysis. Given material amounting to only a few mg. per 100 cc. analysis by the above methods becomes impossible. Using the data of Table I, we find that *Proteus vulgaris* removes glucose, and leaves fructose and sucrose; *Monilia krusei* removes fructose and leaves sucrose. The sucrose can then be hydrolysed by dilute HCl, the solution neutralised, and the total invert

¹ In sugar determinations following the use of *Proteus*, and using the reagent detailed by Harding and Downs, it is essential that the I liberated from the KI be allowed to stand in contact with the Cu_2O for at least 3 mins. with occasional agitation before proceeding with the thiosulphate titration.

sugar determined by *M. krusei*, or the glucose and fructose estimated separately by *Proteus vulgaris* followed by *M. krusei*.

The details of an analysis of a known mixture of glucose, fructose and sucrose are shown in Table II.

Table II. Showing the details of an analysis of glucose, fructose and sucrose.

Glucose	10 mg.	} 100 cc.	$\left. \begin{aligned} &=1.72 \\ &=1.69 \\ &=0.00 \end{aligned} \right\} = 3.41^* \text{ cc. of } 0.005 \text{ N I for } 2.0 \text{ cc. solution}$
Fructose	10 „		
Sucrose	10 „		
cc. 0.005 N I for 2.0 cc. solution			
(1) Total reduction found	...	...	3.41
(2) Reduction after <i>Proteus</i>	...	...	1.70
3.41 - 1.70 = 1.71 = 10 mg. glucose removed by <i>Proteus</i>			
(3) Reduction after <i>M. krusei</i>	...	...	0.01
1.70 - 0.01 = 1.69 = 10 mg. fructose removed by <i>M. krusei</i>			
(4) Add 0.01 cc. N HCl to residual from (3) after removal of <i>M. krusei</i> .			
Heat 30 mins. in boiling water-bath. Add 0.01 cc. N NaOH.			
Total reduction	...	...	1.83
(5) Reduction after <i>Proteus</i> action on (4)	...	...	0.91
1.83 - 0.91 = 0.92 = 5.3 mg. glucose removed by <i>Proteus</i>			
(6) Reduction after <i>M. krusei</i> on (5)	...	...	0.01
0.91 - 0.01 = 0.90 = 5.3 mg. fructose removed by <i>M. krusei</i>			
(7) Equimolecular mixture of glucose and fructose = 10 mg. sucrose in analysis mixture.			

The amounts of wet weight washed organism and the time of incubation are those of Table I.

* Data from Table I [Harding and Downs, 1933].

This analysis is successful in absence of mannose. While mannose does not usually occur in natural mixtures containing glucose, fructose and sucrose, its absence should be established in those of unknown origin. We have found, so far, no biological method for the separation of fructose and mannose. Incorrect results will also be obtained in presence of maltose, as this sugar is partially hydrolysed by the acid.

Under these circumstances either the amount of sucrose can be calculated from the fructose produced by hydrolysis, or the maltose can be removed by *M. pinoyi* previous to hydrolysis. We have obtained satisfactory results by either method. In the presence of galactose a full analysis must be conducted according to the scheme given in the final section.

Application of Proteus to urines. Fasting human urine is cleared with H_2SO_4 and Lloyd's reagent [Folin and Berglund, 1922; Hamilton, 1928; Harding and Selby, 1931] with a final dilution of 1:10. The NH_3 is removed by KH_2PO_4 and MgO , and the total reduction determined. 25 cc. of the Lloyd filtrate are now brought to p_{H} 7.0 by one or two drops of 25 % NaOH . 10.0 cc. of this are added to 0.75 g. wet weight washed *Proteus* in the aluminium centrifuge-tube and incubated 30 mins. at 38° with stirring by means of a thin glass rod. 0.5 g. KH_2PO_4 is then added, the main portion of the cells removed by the high angle centrifuge, and the remainder by filtration through kieselguhr. The NH_3 is now removed by the addition of 0.75 g. MgO with agitation for 30 mins. The solution is brought back to p_{H} 6.5 by H_2SO_4 , and the residual sugar determined. The difference between the two determinations gives glucose in the known absence of galactose. Table III shows the action of *Proteus* in removing added glucose and its inertness towards added fructose, mannose, maltose, arabinose and xylose. We have also used *Proteus vulgaris* on urines cleared by HgSO_4 - BaCO_3 [West and Peterson, 1932]. We have sometimes found a continued action of the *Proteus* urease after centrifuging and the KH_2PO_4 - MgO treatment. The urease is destroyed in acid solution by a few minutes' heating at 70 - 80° .

It will be noted that Table III shows apparent small amounts of glucose (*a-c*) as present in normal fasting urine, whereas Harding and Selby [1931] had been unable to find any fermentable sugar in most specimens.

Table III. *Showing the action of Proteus vulgaris on urine and on urine with added sugar after treatment with Lloyd's reagent.*

Added sugar	Reducing value of urine after NH_3 removal (a)	Reducing value of urine+sugar after NH_3 removal (b)	Value of added sugar in urine after NH_3 removal (b) - (a)	Reducing value of urine (a) after <i>Proteus</i> (c)	Urine-sugar (a) - (c)	Reducing value of urine+sugar (b) after <i>Proteus</i> (d)	Added sugar after <i>Proteus</i> (d) - (c)	% removal of added sugar
Fructose	1.04	2.80	1.76	0.84	0.20	2.61	1.77	0
Arabinose	0.73	2.05	1.32	0.40	0.33	1.72	1.32	0
Lactose	0.65	1.29	0.64	0.49	0.16	1.13	0.64	0
Maltose	0.83	1.52	0.69	0.45	0.38	1.13	0.68	0
Galactose	0.90	2.11	1.26	0.42	0.48	1.62	1.20	4
Glucose	0.97	2.74	1.77	0.73	0.24	0.73	0	100
Xylose	0.89	2.17	1.28	0.48	0.41	1.74	1.26	0
Mannose	1.04	2.10	1.06	0.64	0.40	1.69	1.05	0

The above values are expressed as cc. of 0.005 *N* I for 2.0 cc. urine (1:10) filtrate. For mg. added sugar see Table I [Harding and Downs, 1933].

Application of Proteus to Folin-Wu blood-filtrates. The method given for the estimation of glucose in aqueous solution can be applied to Folin-Wu blood-filtrates. The values obtained with two samples of dog's blood and six normal human fasting bloods as shown in Table IV differ in

Table IV. *Comparison of sugar removal by ordinary yeast and by Proteus vulgaris from Folin-Wu blood-filtrates.*

Blood No.	Total reducing substances	Residual reducing substances after yeast	Residual reducing substances after <i>Proteus</i>	Glucose mg./100 cc.
Dog 1	1.98	0.45	0.44	88
" 2	1.61	0.27	0.28	77
Man 3	1.57	0.28	0.31	73
" 4	1.70	0.35	0.33	78
" 5	1.72	0.35	0.33	79
" 6	1.71	0.34	0.33	78
" 7	1.84	0.40	0.40	83
" 8	1.52	0.40	0.39	66

Figures represent cc. 0.005 *N* I for 2 cc. blood-filtrate.

Table V. *Separation of glucose and non-glucose sugars from fermented Folin-Wu blood-filtrates.*

Value for residual reduction after yeast	Value after added non-glucose sugar	Value after added non-glucose sugar + added glucose	Value after <i>Proteus</i>	Added non-glucose sugar
0.31	0.39	2.07	0.42	Fructose
0.34	0.40	2.21	0.41	Maltose
0.40	0.46	1.79	0.44	Mannose
0.42	0.65	2.37	0.66	Lactose
0.42	0.87	2.53	0.88	Arabinose

Figures represent cc. 0.005 *N* I for 2 cc. blood-filtrate.

no wise from those ordinarily accepted. If "fermentable sugar" is determined by ordinary washed baker's yeast, using 0.25 g. of wet weight yeast for 8 mins. at 38° for 10 cc. of the same Folin-Wu blood-filtrate, the values are identical with those obtained by the use of *Proteus*. Table V shows in a similar manner the recovery of glucose added to Folin-Wu blood-filtrates previously fermented by ordinary yeast, and the inertness of the reagent to small added amounts of fructose, mannose, maltose, lactose and arabinose. The closeness of the residual reduction figures after

Proteus and after baker's yeast supports the use of the latter as a reliable reagent for glucose in normal bloods. In nephritic bloods with a high amount of urea, the use of *Proteus* for the determination of glucose without removal of NH_3 would lead to serious error.

The use of Monilia tropicalis for the estimation of maltose.

Maltose can be identified by its rotation, reducing power and osazone. These also supply data for its determination. In presence of other sugars or reducing material, and in small quantities, its estimation becomes one of difficulty. Barfoed's solution estimates monosaccharides in presence of disaccharides [Legrand, 1921; Tauber and Kleiner, 1932-33]. If the only disaccharide present is maltose, the difference between the monosaccharide reduction and the total reduction gives a measure of the maltose, but such estimations are obviously limited in application. Archbold and Widdowson [1931], in studying the sugar changes during apple storage, have utilised the differing rates of oxidation by Hanes's [1929] ferricyanide solution, and by hypiodite, for the simultaneous estimation of maltose and glucose. Smith [1932] has made use of the increased reducing power on hydrolysis as a basis of estimation of the same two sugars.

We have found no yeast which possesses a fermentative action on maltose alone. In Table I, however, are listed four organisms without any action on maltose and two organisms capable of its quantitative removal. Either of these last two (*M. pinoyi* or *M. tropicalis*), could be used as an analytical reagent. Of the two, *M. tropicalis* is the more active. (0.5 g. of the centrifuged freshly grown and washed cells remove 2-3 mg. maltose from 10 cc. liquid in 8 mins. at 38°.) Its action is slower on fructose and mannose, requiring 20 mins. for complete removal of the same quantity. *M. pinoyi* requires 30 mins. incubation to remove the same amount of maltose and is not quantitative in its action on fructose and mannose under the same conditions. *M. tropicalis* is thus preferable, though *M. pinoyi* may be used to separate maltose and saccharose. If *M. tropicalis* is preceded by any one of the four organisms having no removal action on maltose (*Proteus vulgaris*, *M. krusei*, *M. macedoninensis*, or *S. marxianus*) other sugars may be partially or completely removed. The use of *S. marxianus* followed by *M. tropicalis* gives a method specific for the estimation of maltose.

Culture and preparation. Inoculate a 2 % glucose-peptone broth, phosphate-buffered to p_{H} 5.5, with the organism, and incubate for 24-48 hours at 38°. Place 5-7 cc. of the 24-hour broth culture on 2 % neutral glucose-agar in a 26 oz. narrow-necked bottle, and grow for 24 hours at 38°. Wash off the growth with distilled water. Centrifuge at 1500-2500 r.p.m. Wash several times with water until the washings are free from reducing material. Yield, about 2 g. wet-weight of yeast per bottle. The organism is made to a 50 % solution with water and is used within 12 hours. The culture is controlled by microscopical examination for bacterial contamination. Every 4-5 weeks a new culture is prepared from a single colony picked from an agar-plate growth. The new culture should be tested against a series of sugars.

Estimation of maltose. (a) The total reducing power of 2.0 cc. sugar solution is determined. The sugar solution should not contain an amount greater than the equivalent of 20 mg. glucose per 100 cc. [see Table I, Harding and Downs, 1933].

(b) If sugars other than maltose are known to be present 12.0 cc. of the solution are placed in a centrifuge-tube, containing 0.5 g. wet weight washed *Saccharomyces marxianus* [Harding, Nicholson and Grant, 1932-33]. Incubate for 30 mins. at 38° with stirring. The *S. marxianus* is removed by centrifuging, taking care to remove completely any stray cells. The reducing power of 2.0 cc. of the centrifugate is determined. A control tube for the presence of small amounts of reducing material in the yeast is made under similar conditions with water instead of the sugar solution. The reducing value is corrected. The difference $a-b$ represents the reducing action of glucose, fructose, mannose or galactose, either singly or in combination.

(c) The remainder of the centrifugate from (b) (5-6 cc.) is added to a centrifuge-tube containing 0.5 g. wet-weight *M. tropicalis*. This latter has previously been added from the fresh 50 % suspension, the excess water has been removed by centrifuging and the interior of the tube wiped dry with filter-paper. The *Monilia* and sugar solution are mixed with a thin glass rod, or by tapping, incubated for 20 mins. at 38° with occasional stirring and then separated by centrifuging. The reducing power of 2.0 cc. of the centrifugate is then determined. Care is taken that no stray cells remain, previous to the "sugar" determination. A control tube with water and *M. tropicalis* is made simultaneously. The difference *b-c* represents maltose.

Application to blood and urine. Folin-Wu blood-filtrates were deprived of glucose by ordinary yeast, and to them were added a series of sugars. The removal of the added sugars by *M. tropicalis* is the same as in water and shows the same accuracy. Filtrates from dog's blood also gave similar removal figures.

The action of *M. tropicalis* on urine, either alone or in presence of added sugar has been tested as in testing the action of *Proteus*, except that no removal of NH_3 after fermentation is required. *M. tropicalis* removes glucose, fructose, mannose and maltose quantitatively. The removal of galactose from urine appears to be greater than from water solution. There is no action on lactose, arabinose and xylose.

*Use of baker's and brewer's yeasts for the separation of
glucose and maltose.*

Raymond and Blanco [1928, 1] found no removal of maltose by baker's yeast. Our observations on ordinary baker's yeast have convinced us that its maltose removal power is very variable, especially if 0.5 g. wet weight yeast is used and the incubation period at 38° is extended to 30 mins. On 10 cc. of a 20 mg. per 100 cc. solution of maltose, the disappearance of the sugar ranged from 16 to 77 % under such conditions. On the other hand, using 0.25 g. baker's yeast and incubating for 8 mins. at the same temperature, the removal of maltose was sometimes nil. As Raymond and Blanco only incubated for 3 mins., it is easy to see how they might fail to observe the disappearance of maltose. How complete can be the separation of glucose and maltose by the use of a small amount of yeast, coupled with a short period of incubation, can be seen from Table VI. Within experimental error and from a range of 1.25 to 10 mg.

Table VI. *Showing the action of 0.25 g. washed baker's yeast on maltose
and glucose mixtures after incubation for 8 mins. at 35°.*

Sugars		cc. 0.005 N I* for 2 cc. sugar solution	
Maltose mg./100 cc.	Added glucose mg./100 cc.	Original reduction	Reduction after yeast
20	0	1.23	0.84
10	0	0.62	0.60
5	0	0.31	0.29
2.5	0	0.16	0.15
1.25	0	0.07	0.07
20	1	1.39	0.92
10	1	0.78	0.60
5	1	0.47	0.30
2.5	1	0.32	0.15
1.25	1	0.24	0.07
20	20	4.68	1.10
10	20	4.03	0.63
5	20	3.75	0.31
2.5	20	3.59	0.13
1.25	20	3.50	0.08

* The iodide-free Shaffer-Hartmann reagent described by Harding and Selby [1931] was used in this experiment.

per 100 cc. the ordinary yeast cake (Fleischmann), not only had no action on maltose, but completely removed added glucose in concentrations of 1–20 mg./100 cc. It is to be noted, however, that at a higher concentration of 20 mg./100 cc. the yeast removed considerable quantities of maltose. The highest removal of maltose noticed by us with baker's yeast followed the use of a special sample, packed in ice by the laboratories of the Standard Brands Inc., and sent direct by express.

Two other observations convinced us that the freshness of the yeast was an important factor in the production of a high maltose-removal power. Cultures of baker's yeast grown in glucose-peptone broth, followed by growth on glucose-agar removed the maltose quantitatively. Brewer's yeast obtained fresh from the vat, and used immediately, also accomplished a 100 % removal of maltose, but at the end of 3 days the removal power had dropped to 64 %. Our standard criterion of removal in these experiments was the loss of reducing power experienced by 10.0 cc. of a 20 mg./100 cc. maltose solution when incubated for 30 mins. at 38° with 0.5 g. of wet-weight washed yeast.

To obtain baker's yeast with a very low maltose removal power required a number of experiments. Although it was evident that on standing the yeast lost some of its action, simple slow autolysis at 4°, or at room temperature, never yielded a product sufficiently low in maltose-removal power to be dependable as an analytical reagent. The effect of added phenol, or of desiccation at 38°, was to lower the maltose-removal power more rapidly, but attempts to accelerate these effects led to the death of the cell. Finally the simple expedient of allowing the small package cakes of yeast, as bought, and still wrapped in the foil covering, to remain on the laboratory bench for 7 days proved successful. The process is probably one of autolysis combined with slow desiccation. The packages were turned over daily. Any discoloured portions were removed before testing the action on sugars. The results of such an experiment and the effect of the subsequent reculture on the maltose-removal power are shown in Table VII. In both samples

Table VII. *Effect of "ageing" of baker's yeast on sugar removals. Figures show percentage removal produced by 0.5 g. yeast on 2 mg. sugar in 10 cc. solution at 38°.*

Time of incubation in mins....	Yeast Sample I						Yeast Sample II								
	After 7 days*			Fresh culture			As bought			After 7 days			Fresh cultures		
	8	20	30	8	20	30	8	20	30	8	20	30	8	20	30
Glucose	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Fructose	72	95	100	67	96	100	80	95	100	73	99	100	83	—	100
Mannose	88	97	100	—	—	—	—	—	—	—	—	100	—	—	100
Maltose	4	—	6	91	100	100	16	19	32	2	—	11	62	86	100
Saccharose	79	96	100	—	—	—	64	97	100	95	98	100	94	96	100

* Removal power of original yeast cakes undetermined.

the maltose-removal power is very low at the end of 7 days. The use of such yeast preparations acting, in small amount, for a short period of time gives a negligible removal of maltose. On the other hand the preparations are still active towards glucose, fructose, mannose and sucrose. Too long an exposure results in the death of the cell. The length of time of "ageing" must evidently vary from laboratory to laboratory, depending on temperature, humidity *etc.*

The foregoing experiments point to procedures for the analysis of mixtures of glucose and maltose. 0.25 g. wet-weight washed baker's yeast prepared from the "aged" partially desiccated packages and incubated for 3–8 mins., at 38° completely removes 2 mg. glucose. 0.5 g. wet-weight washed active brewer's yeast or baker's yeast just removed from a laboratory fermentation incubated 30 mins. at 38° completely removes 2 mg. maltose.

In view of possible variations each preparation of glucose-removing and maltose-removing baker's yeast should be tested on known mixtures of the two sugars. The experiments also indicate

one of the sources of the discrepancies sometimes encountered between different laboratories in the use of yeast for the separation of fermentable and non-fermentable sugars. Also, the strains or mixtures of yeasts used in commercial yeast cakes vary and contribute to failure of duplication of results. They emphasise the necessity of pure cultures.

Use of Monilia krusei.

M. krusei is an important organism from the standpoint of sugar analysis. It is the only one of our series which has no removal action on galactose at 20 mg./100 cc. concentration and has proved quite reliable in the separation of mixtures of glucose, fructose and mannose from that sugar. We have already noted that from higher concentrations of galactose, a small fraction is removed. In a previous section we have illustrated its use in the analysis of mixtures of glucose, fructose and sucrose. The cultural conditions of *M. krusei* are the same as for *M. tropicalis*.

M. krusei has the same removal reaction on sugars in Folin-Wu blood-filtrates and urines cleared with Lloyd reagent as in water.

Analysis of mixtures of sugars.

From the foregoing results and those of a previous paper [Harding, Nicholson and Grant, 1932-33] it is possible to construct a system of sugar analysis. The use of *Proteus vulgaris*, *M. krusei*, *S. marxianus* and *M. tropicalis*, combined with acid hydrolysis at appropriate stages, enables one to analyse a mixture of glucose, fructose or mannose, galactose, sucrose, maltose and lactose. A summary of such an analysis shows the following steps:

- A. Estimate *glucose, fructose, mannose* by *M. krusei*.
- B. Estimate *galactose* on residual fluid from A by *S. marxianus*.
- C. (In absence of galactose) estimate *glucose* by *Proteus vulgaris*.
 $A - C = \text{fructose-mannose.}$
- D. (In presence of galactose) estimate *fructose-mannose* on residual fluid from C by *M. krusei*.
 $A - D = \text{glucose.}$
- E. Estimate *sucrose* on residual fluid from A or D by hydrolysis, followed by use of *Proteus vulgaris* and *M. krusei*. (In presence of galactose or maltose calculate the sucrose from the fructose value.)
- F. Estimate *maltose* by *S. marxianus* followed by *M. tropicalis*.
- G. Estimate *lactose* on fluid from F by hydrolysis. Calculate the lactose from the galactose value, assuming 72 % hydrolysis [Harding and Grant, 1931].

The estimations A, C, F are made on the original mixture.

The details of analysis of the six sugars, each present at a concentration of 10 mg./100 cc. have been given in the preliminary account of this work [Harding *et al.*, 1932]. The percentage recovery of the sugars is shown in Table VIII.

Table VIII. *Showing percentage recovery from mixtures of sugars, analysed by the biological reagents.*

	I	II
Glucose	97.6	95.4
Fructose	101.7	101.1
Galactose	96.7	100.8
Sucrose	103.0	103.0
Maltose	109.6	105.1
Lactose	98.8	106.2

All four biological reagents act in aqueous solution at p_H 6.5–7.0. The cultural conditions must be strictly followed, otherwise considerable variations in the removal power are encountered. The application to biological fluids must always be accompanied by a considerable number of control experiments, in which the efficacy and specificity of the organisms are demonstrated. The number of sugars examined, while adequate for our present purposes, is not exhaustive. Reducing substances other than sugars may be removed, or have their reducing properties diminished. The presence in biological fluids of substances considerably modifying or even radically altering, the removal action of the organisms must be considered. Our own experience with *Proteus* on urine, where its action on the urea forms sufficient NH_3 to interfere with the accuracy of the copper reagent, illustrates another possible error. Used carefully, and with due regard to possible error, we believe biological reagents, such as we have described, will be of value in the study of many phases of carbohydrate metabolism, both in animal and vegetable physiology. Used indiscriminately, and without due regard to their limitations, they will prove inferior to the classical but slower methods of chemical identification of the sugars.

The possibility of such errors is reduced, if some removal of non-sugar material preliminary to the sugar estimation is adopted. Or the sugars may be separated in some insoluble combination from the other biological material and then liberated in aqueous solution. Under both sets of circumstances the absence of more than mere traces of heavy metals must be ensured. We have used the organisms after treatment of biological material by the following:

- (1) Lead acetate, followed by potassium dihydrogen phosphate.
- (2) Potassium dihydrogen phosphate followed by solid magnesium oxide.
- (3) Mercuric sulphate and barium carbonate followed by hydrogen sulphide [West and Peterson, 1932].
- (4) Copper sulphate and lime, with liberation of the sugar by sulphuric acid and hydrogen sulphide.

SUMMARY.

A number of micro-organisms have been examined as possible analytical reagents for sugars.

A close relationship between sugar removal power and fermentation has been shown.

Four of the organisms examined appear useful as biological reagents for sugars.

A strain of *Proteus vulgaris* has been developed as an analytical reagent for glucose.

Proteus is without removal action on fructose, mannose, maltose, lactose, sucrose, arabinose and xylose, but is variable towards galactose.

Proteus can be applied to Folin-Wu blood-filtrates and to urines after treatment with H_2SO_4 and Lloyd's reagent and after treatment with $HgSO_4$ and $BaCO_3$.

Details of the analysis of mixtures of glucose, fructose and sucrose are given.

Monilia tropicalis is extremely active in removal of maltose.

A method for the estimation of maltose is given, depending on the use of *Saccharomyces marxianus*, followed by *Monilia tropicalis*.

Baker's and brewer's yeasts show variation in their removal power towards maltose, depending on the freshness of the organism.

A method using "aged" and "fresh" baker's yeast for the separation of glucose and maltose is suggested.

Monilia krusei is a useful sugar reagent, as it only removes glucose, fructose and mannose.

A system of carbohydrate analysis, embracing glucose, fructose-mannose, galactose, sucrose, maltose and lactose, is outlined, and the result of the analysis of such a mixture by the biological reagents is given.

Some precautions in the use of organisms as analytical reagents for sugars are suggested.

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REFERENCES.

- Archbold and Widdowson (1931). *Biochem. J.* **25**, 101.
Benedict (1928). *J. Biol. Chem.* **76**, 464.
Castellani and Taylor (1917). *Brit. Med. J.* ii, 855.
— (1919). *Brit. Med. J.* i, 183.
— (1922). *Biochem. J.* **16**, 655.
Day, Gibbs, Walker and Jung (1930). *J. Infect. Dis.* **47**, 490.
Folin and Berglund (1922). *J. Biol. Chem.* **51**, 213.
Hamilton (1928). *J. Biol. Chem.* **78**, 63.
Hanes (1929). *Biochem. J.* **23**, 99.
Harding and Downs (1933). *J. Biol. Chem.* (In press.)
— and Grant (1931). *J. Biol. Chem.* **94**, 529.
— Nicholson and Grant (1932–33). *J. Biol. Chem.* **99**, 625.
— — — — — Hern and Downs (1932). *Trans. Roy. Soc. Can.* **26** (v), 33.
— and Selby (1931). *Biochem. J.* **25**, 1815.
Hiller, Linder and Van Slyke (1925). *J. Biol. Chem.* **64**, 625.
Höst and Hatlehol (1920). *J. Biol. Chem.* **42**, 347.
Kendall (1923). *J. Infect. Dis.* **32**, 362.
— and Yoshida (1923). *J. Infect. Dis.* **32**, 369.
Larson (1932). *J. Biol. Chem.* **98**, 151.
Legrand (1921). *Compt. Rend. Acad. Sci.* **172**, 602.
MacLeod (1921). *Physiol. Rev.* **1**, 208.
Raymond and Blanco (1928, 1). *J. Biol. Chem.* **79**, 649.
— (1928, 2). *J. Biol. Chem.* **80**, 631.
Schrumpf (1932). *Scand. Arch. Physiol.* **65**, 1.
Slater and Sand (1910). *J. Chem. Soc.* **97**, 922.
Smith (1932). *Biochem. J.* **26**, 1459.
Somogyi (1927). *J. Biol. Chem.* **75**, 33.
Tauber and Kleiner (1932–33). *J. Biol. Chem.* **99**, 249.
Van Slyke and Hawkins (1929). *J. Biol. Chem.* **83**, 51.
West and Peterson (1932). *Biochem. J.* **26**, 1720.