76. THE USE OF MICRO-ORGANISMS IN SUGAR ANALYSIS

3. THE EFFECTS OF VARIOUS SALTS ON THE ACTIVITY OF *PROTEUS VULGARIS* IN REMOVING GLUCOSE AND SOME POSSIBLE SOURCES OF ERROR IN ITS USE AS A REAGENT FOR THE ESTIMATION OF GLUCOSE

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IN the eight years since *Proteus vulgaris* was described as a reagent for the quantitative estimation of small amounts of glucose [Harding & Nicholson, 1933], continued experience has revealed a number of possible sources of error, and improvements in the methods of growth and use of the organism have simplified the procedure.

The effect of salt concentration

It was noted previously [Harding *et al.* 1936] that a concentration of 5% Na_2SO_4 inhibited completely the glucose-removing action of *P. vulgaris* and that a concentration of 3% led to a definite decrease in activity. The effects of varying concentrations of different salts which might be encountered in filtrates or hydrolysates of biological materials were determined. The results are shown in Table 1.

Table 1. *The effect of salt concentration on the glucose-removing power of P. vulgaris*

Molar concentration	% removal*					
	Na_2SO_4	K_2SO_4	NaCl	KCl	K_2HPO_4 $\text{KH}_2\text{PO}_4^\dagger$	Na_2HPO_4 $\text{NaH}_2\text{PO}_4^\dagger$
0.00	100	100	100	100	100	100
0.0075	—	—	—	—	350	—
0.015	112	130	119	142	350	231
0.02	110	128	123	158	—	—
0.03	110	124	132	188	350	231
0.05	86	119	140	184	—	—
0.07	70	104	135	180	350	230
0.15	43	63	103	148	350	164
0.20	30	52	85	114	—	74
0.25	24	43	73	89	350	21
0.35	0	32	53	63	—	—
0.45	—	31	34	55	—	—
0.55	—	31	23	50	—	—
0.65	—	30	17	46	—	—
0.75	—	29	—	—	—	—
1.50	—	—	16	44	350	20

* The amount of *P. vulgaris* used was just sufficient to remove 20% of the glucose from 10 ml. of a 20 mg. per 100 ml. solution in 30 min. at 37°. This removal was taken as 100 for purposes of comparison.

† These mixtures were made up so that the concentrations were of 0.918 *M* of the dibasic salt and 0.588 *M* of the monobasic salt in the stock solution. The pH of the various dilutions ranged from 6.9 in the strongest to 7.1 in the weakest solution.

Small concentrations of all the salts tested had an accelerating effect on the action of *P. vulgaris*. Potassium salts had a greater effect than sodium salts. Chlorides were more active than sulphates. At higher concentrations there is a marked inhibition of the removal activity, complete in the case of sodium sulphate at a concentration of 0.35 *M* and reaching a fairly constant level for each of the other salts tested: 30% removal at concentrations of 0.35 *M* and over for K_2SO_4 : 16% removal at concentrations of 0.65 *M* and over for NaCl: 46% removal at the same concentration or over for KCl. The sodium phosphate mixtures had a very similar effect, except that the activating effect of the lower concentrations was more marked. Potassium phosphate mixtures acted quite differently in that the activation was much more marked and was present throughout the range of concentrations employed.

The inhibiting effect of the higher salt concentrations has also been observed with *Gaffkya tetragena*, an organism which is used as a reagent for the removal of glucose and fructose [Nicholson, 1936], but the lower concentrations produce no increase in activity. This applies to the phosphates as well as to the other salts. No change whatever in sugar-removing power has been observed in the case of yeasts.

The marked activating power of potassium phosphate is not due in more than a minor degree to any buffering action, as is shown in Table 2.

Table 2. *Effects of phosphate, bicarbonate-carbonic acid and hydroxymethylglyoxaline hydrochloride-NaOH buffers on the activity of P. vulgaris in removing glucose from a solution containing 2 mg. glucose*

	No salt —	KH_2PO_4 - K_2HPO_4 0.03 <i>M</i>	$NaHCO_3$ - * H_2CO_3 0.03 <i>M</i>	KCl 0.03 <i>M</i>	4 (or 5)-hydroxymethyl- glyoxaline hydro- chloride + NaOH† [Kirby & Neuberger, 1938]
mg. glucose removed	0.28	1.0	0.56	0.43	0.53

All fermentations were carried out at a pH of 7.0. Removal in mg. from 10 ml. of a 10 mg. per 100 ml. solution of glucose using 0.10 g. *P. vulgaris* and incubating for one half-hour at 37–38°.

* A slow stream of CO_2 was run through the solution during incubation.

† We are indebted to Dr H. O. L. Fischer of the Department of Chemistry of this University for the dihydroxyacetone from which the hydroxymethylglyoxaline hydrochloride was prepared.

The glucose-removing activity of *P. vulgaris* may be further increased by adding potassium phosphate to the media in which the organisms are grown.

The following media have been used:

Phosphate broth: 4375 ml. tap water, 625 ml. phosphate solution (see below), 50 g. NaCl, 30 g. glucose, 100 g. bacto-peptone (Lemco 62). Sterilize and tube in the usual manner.

Phosphate agar: 30 g. agar, 875 ml. broth, 50 ml. phosphate solution (see below), 10 g. glucose. Sterilize and bottle.

The potassium phosphate may be most conveniently added during the course of the dilution of the solution to be analysed for glucose. If, however, a stock 1.5 *M* phosphate mixture (16 g. K_2HPO_4 + 8 g. KH_2PO_4 in 100 ml. water) is used, the dilution (0.5%) involved in adding the potassium phosphate to the actual solution being fermented is negligible.

The batches of *P. vulgaris* grown on such media showed 60% greater activity than did those grown on the media originally described [Harding & Nicholson, 1933]. When *P. vulgaris* grown on phosphate-containing media is used on glucose solutions containing potassium phosphate (0.0075 *M*), the glucose-removing power of the organisms is so increased that 0.5 g. of wet-

weight packed organisms will remove 2.5 mg. of sugar by incubation for 10 min. at 37°. When grown on the original media and acting without phosphate added to the glucose-containing solution it takes 0.5 g. wet-weight packed bacteria and incubation for half an hour at 37° to remove 1 mg. of glucose. These procedures have no effect on the specificity of *P. vulgaris* for glucose.

The accelerating action of phosphates on yeast juices and maceration extracts is well known, as is the effect of higher concentrations in yeast-juice fermentation in delaying the rate of CO₂ production [Harden & Young, 1908; Harden, 1932]. Meyerhof [1918] noted the retarding effect of salts other than phosphates and Harden & Henley [1921] observed that sulphates had a greater retarding effect than chlorides. In the case of *Proteus*, however, the accelerating effect of phosphate is noted in the intact organism, not in its extracts only as in the case of yeast. Most of the observations on the retardation effects of salts on yeast have been made on extracts, but Lampe & Kilk [1931] noted that the NaCl formed by the neutralization of HCl used in the inversion of cane sugar decreased the alcohol yield on fermentation. The action of *P. vulgaris*, however, is retarded more by salts of sodium than by those of potassium, whereas yeast extracts are affected equally by either [Harden & Henley, 1921]. In the case of *P. vulgaris* there is also an accelerating effect by small concentrations of chlorides and sulphates as well as phosphates and the potassium phosphate has no retarding effect even in relatively high concentrations.

The effect of pH

It was previously reported [Harding & Nicholson, 1933] that *P. vulgaris* would not act on glucose at a pH lower than 6.5 or higher than 8.0. It has been found, however, that it is inadvisable to use this micro-organism at any pH higher than 7.2 because, although the bacteria are still active in removing glucose in the more alkaline solutions, there is a small amount of lysis with the liberation of varying amounts of reducing substances. If *Proteus* alone is being used this does not affect the accuracy of the glucose estimation, provided that the blank is incubated at the same pH as the solution being tested, but if a differential fermentation is performed it is essential that the reaction be kept below pH 7.2, preferably between pH 6.5 and 7.0 because some of the products of the bacteriolysis are acted on by yeasts to give reducing products. This action is illustrated in Table 3.

Table 3. *Action of P. vulgaris followed by Monilia Krusei on a solution containing 4 mg./100 ml. glucose and 4 mg./100 ml. fructose (a) in neutral solution (pH 7.0), (b) in alkaline solution (pH 7.8)*

Total reduction	<i>P. vulgaris</i>			<i>P. vulgaris</i> followed by <i>M. Krusei</i>		
	Blank	Reduction after fermentation	Removal	Blank	Reduction after fermentation	Removal
(a) 0.69	0.00	0.34	0.35	0.02	0.02	0.34
(b) 0.69	0.09	0.43	0.34	0.17	0.72	Added 0.21

The reducing values were determined using the Harding & Downs [1933] reagent. 1 ml. of reagent and 1 ml. of sugar solution were used. The results are expressed as ml. of 0.005 N Na₂S₂O₃.

This phenomenon is of particular importance in solutions containing any considerable amount of urea, because *P. vulgaris* will produce sufficient ammonia from the urea to make the solutions quite alkaline. Such solutions must therefore be well buffered before the introduction of *P. vulgaris*, or preferably be freed from urea.

The effect of the presence of nitrates

P. vulgaris converts nitrates into nitrites. The nitrites will liberate I_2 from KI in acid solution and therefore interfere with any iodimetric sugar estimation. The sugars may be obtained free from nitrates and other interfering substances by the modified copper-lime technique [Harding *et al.* 1936], or the nitrites may be converted into NH_3 by saturating the solution with H_2S and shaking vigorously in presence of air for a few minutes. The H_2S is then removed by aeration; the NH_3 is removed by the use of KH_2PO_4 and MgO [Harding & Downs, 1933].

Some modifications in the methods of using P. vulgaris

The great enhancement of the glucose-removing power of *P. vulgaris* in the presence of potassium phosphate together with the use of Lloyd's reagent for final clearing of the *P. vulgaris* from the fermented solutions after centrifuging [Griffiths & Waters, 1936] has led to the adoption of the following procedure. The organisms are washed off the phosphate agar surface, packed in the angle centrifuge, then rewashed once in distilled water. A 25 % suspension of the wet packed bacteria is made in distilled water. 2 ml. of the suspension (containing 0.5 g. *P. vulgaris*) are taken for each 2.0 mg. of glucose to be removed and the organisms are packed and dried as described by Harding & Nicholson [1933]. Add to each 10 ml. of the sugar solution to be tested, preferably during dilution, 0.05 ml. of a solution of potassium phosphate:

Incubate in the water bath at 37–38° for 10 min. and centrifuge. Clear the few remaining organisms from the supernatant fluid by shaking with a little Lloyd's reagent and filtering through a fluted filter paper [Griffiths & Waters, 1936].

If no angle centrifuge is available the following procedure may be followed. Wash the bacteria from agar, place not more than 7 ml. of the suspension in a 15 ml. conical centrifuge tube and centrifuge at 2500 revolutions per min. for 15 min. Pour off the cloudy supernatant fluid. Add 5 ml. of distilled water, resuspend and recentrifuge. Pour off cloudy supernatant and recentrifuge for 15 min. Make up a 50 % suspension of the bacteria. Put not more than 0.5 ml. of the suspension (sufficient to remove 1 mg. glucose in 10 min.) in a 15 ml. centrifuge tube, centrifuge for 15 min., pour off the supernatant fluid and recentrifuge for 5 min. Pour off any fluid remaining and dry the sides of the centrifuge tube. Incubate with the solution to be tested (containing not more than 1 mg. glucose) in the presence of 0.0075 *M* phosphate. After incubation centrifuge for 15 min. Clear the markedly cloudy supernatant fluid by shaking with 1.2 g. Lloyd's reagent and filtering.

SUMMARY

The ability of *P. vulgaris* to remove glucose from solution is enhanced by small concentrations of NaCl, Na_2SO_4 , KCl and K_2SO_4 and is greatly inhibited by larger concentrations.

The potassium salts are the more active accelerators and sodium salts are the stronger inhibitors. Sulphates show a greater inhibiting effect than do chlorides.

Sodium phosphate in small concentrations and potassium phosphate in all concentrations greatly enhance the glucose-removing ability of the organisms, potassium phosphate increasing the glucose removal 3.5 times.

Fermentation in a solution more alkaline than pH 7.2 introduces errors due to the dissolving of reducing substances from the cells.

The reduction of nitrates to nitrites by *P. vulgaris* may lead to errors in iodimetric titrations. Methods for obviating this difficulty are described.

A method for using *P. vulgaris* without an angle centrifuge is given.

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