of absorption in the ultra-violet indicating that these compounds have similar structures.

In the alkaline solutions, both alcoholic and aqueous, the type of absorption in the ultra-violet is different from that of the neutral solutions showing that the phthaleins have undergone a change in constitution. The alkaline solutions, with the exception of tetraiodo- and tetrabromophenolphthaleins, have decided color and in these two cases the apparent absence of color is probably due to the thinness of layer and dilution of the solution necessary to allow the transmission of the ultra-violet light.

The absorption of the alkaline solutions, in the visible region is of the same type for all solutions indicating a similarity in constitution of the salts in solution. As was found in the cases of fluorescein and its halogen derivatives[1] and of the orcinolphthaleins and their halogen derivatives,[2] the introduction of halogens into the molecule shifts the absorption bands toward the red end of the spectrum and the shift is approximately proportional to the number of the halogen atoms introduced rather than to the molecular weight of the substituents.

Summary.

The results of this investigation may be briefly stated as follows:

1. Pure tetraiodophenolphthalein has been prepared and studied. It has been shown not to form a carbinolcarboxylic acid, but colorless salts of such an acid have been obtained. When these salts are heated they lose water or water and alcohol and give the colored salts of the phthalein.

2. A colorless diacetate and dibenzoate and a colored oxime have been made.

3. Tetraiodophenoltetrachlorophthalein has been made and studied. This compound is colorless and yields a colorless diacetate and dibenzoate.

4. The action of ammonia on these 2 phthaleins has been investigated.

5. The absorption spectra of phenolphthalein and some of its halogen derivatives have been studied and the absorption curves plotted.

[Contributions from the Department of Chemistry, Columbia University, No. 310.]

A STUDY OF THE GLUCOSAZONE REACTION.

By Ira D. Garard and H. C. Sherman.

Received February 4, 1918.

Introduction.

Since Fischer[3] first carried out the osazone reaction in 1884, it has been applied to analytical problems by numerous workers, among whom may be

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[1] This Journal, 36, 680 (1914).

[2] *Ibid.*, 37, 1201 (1915).

[3] Fischer, *Ber.*, 17, 579 (1884). See also *Ibid.*, 20, 821 (1887).

mentioned Scheibler,[1] Jacksch,[2] Laves,[3] Maquenne,[4] Lintner and Kröber,[5] Mulliken,[6] and Sherman and Williams.[7] In his first paper, Fischer[8] pointed out that the reaction is a better test for dextrose than the common alkaline metal reductions. The later workers have varied the conditions more or less and made various applications of the reaction.

Few attempts have been made to study the reaction except for specific cases which confronted the various workers, and even within these limits the results reported in the literature are not always concordant.

The purposes of the present work are to determine definitely and somewhat fully the conditions affecting the glucosazone reaction, and the effect of some substances other than glucose; and if possible to improve the method for the detection of glucose, especially when it is present in very small amount with a large amount of some other carbohydrate.

Experimental.

Apparatus, Reagents and Procedure. Phenylhydrazine.—This was used in the form of the free base. It was prepared from the hydrochloride by the addition of saturated sodium hydroxide and then extraction several times with ether. The ethereal solution was dried over solid sodium hydroxide, decanted and the ether removed by distillation on a water bath. The phenylhydrazine was then distilled under a pressure of 20–30 mm. The reagent was prepared in 30 g. lots, since it does not keep when exposed to air.

Glucose.—Merck's "Highest Purity" glucose was used; upon analysis it showed

Moisture, 0.05%; ash, 0.01%; $[\alpha]_D$, 52.42°.

Maltose.—Merck's "Pure" maltose was obtained. It gave $[\alpha]_D$, 130.6°; reducing power, 97.92%; moisture, 0.63%; and so further purification was necessary. The method of Baker and Day[9] was used. The maltose was dissolved in a small volume of water, cooled and poured into enough 95% alcohol to give a final concentration of 85%. It was then filtered, seeded and stirred occasionally for 2 days, when it had crystallized. The process was repeated and the product dried at 70° in a vacuum oven.

$[\alpha]_D$, 137.1°; moisture, 0.4%; ash, 0.065%.

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[1] Scheibler, *Ber.*, **17**, 1729 (1884).

[2] Jacksch, Vaubel's *Bestimmung organisher Verbindung*, **2**, 304 (1902); *Z. klin. Med.*, **11**, 20; *Z. anal. Chem.*, **24**, 478 (1885).

[3] Laves, *Z. anal. Chem.*, **33**, 226 (1894); *Arch. Pharm.*, **231**, 366 (1893).

[4] Maquenne, *Compt. rend.*, **112**, 799 (1891).

[5] Lintner and Kröber, *Z. gesammte Brauwesen*, **19**, 153 (1895).

[6] Milliken, *Identification of Pure Organic Compounds*, Vol. I.

[7] Sherman and Williams, THIS JOURNAL, **28**, 629 (1906).

[8] Fischer, *Loc. cit.*

[9] Baker and Day, *Repts. Brit. Assoc. Adv. Sci.*, Dublin, **1908**, p. 671.

Lactose.—Merck's lactose was recrystallized from hot water, $[\alpha]_D$, 52.3°.

Sucrose.—Well crystallized granulated sugar, $[\alpha]_D = 66.4°$, was used.

Acetic Acid.—Kahlbaum's "C. P." acid was used without further purification.

Sodium Acetate.—"C. P." sodium acetate was used.

Apparatus.—All experiments were carried out in 50 cc. beakers, which were 7 cm. high and about 4 cm. in diameter. These were closed with a cork through which there was a hole about 2–3 mm. in diameter. The small beakers were suspended by means of a wire holder in a liter beaker of boiling water.

The melting points were taken by means of a capillary tube attached to a thermometer in a Thiele melting-point tube filled with conc. sulfuric acid.

All quantitative apparatus used was first calibrated.

Procedure.—Determinations made by Lintner and Kröber,[1] by Maquenne,[1] and by Williams[2] were checked in some cases, but in general a greater yield was obtained. It was found that the literature very seldom mentions the purity of the sugar used and in most cases the conditions were described inadequately. As was found later, the reaction is not complete in 3 hours. For these reasons, it is very difficult to obtain concordant results which check those recorded in the literature. After these observations, an attempt was made to standardize the conditions of the reaction.

The purity of the osazone precipitate was determined from its melting point. Since the melting point of phenylglucosazone is reported from 205° to 230°,[3] it was necessary to establish this constant. Two methods of purification were used: (*a*) that of Fischer, according to which a sample was recrystallized from 60% alcohol, by heating, filtering and cooling; (*b*) that of Tutin,[4] following which the osazone was dissolved in pyridin, and then alcohol, and finally water was added to precipitate it. After 3 recrystallizations by either method, the melting point was 207.9° when the temperature was raised 1° in 2 to 3 seconds as recommended by Fischer.[5] He obtained 208.0°, but Tutin had maintained that when pyridin was used for the recrystallization the value was 217.0°. All melting points taken in this work are given "corrected" as the room temperature and apparatus have a large influence on the result as read.

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[1] *Loc. cit.*

[2] Williams, Unpublished.

[3] LeCoff, *Compt. rend.*, **127**, 817 (1898).

[4] Tutin, *Proc. Chem. Soc.*, **23**, 250 (1907).

[5] Fischer, *Ber.*, **41**, 73 (1908).

Concentration of the Reagent.—In order to establish the concentration of the reagent necessary to produce a maximum yield of osazone a series of determinations were made. In each case a sample of 0.2 g. glucose in a total volume of 20 cc. was heated for 1.5 hours at 100°. The precipitate was collected on a paper in a Gooch crucible, washed with five 10 cc. portions of water at room temperature, dried at 100° and weighed. 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 g. of phenylhydrazine with an equal weight of glacial acetic acid in each case was used and 4 of the determinations in the vicinity of the maximum were repeated. From the results which are shown graphically in Fig. 1, it is seen that a maximum is reached when the concentration of phenylhydrazine lies between 1.0 and 1.2 molar, *i. e.*, about 2.0 g. in 20 cc. From this it falls off until at 1.6 *M* it is only 42.4% of the theoretical.

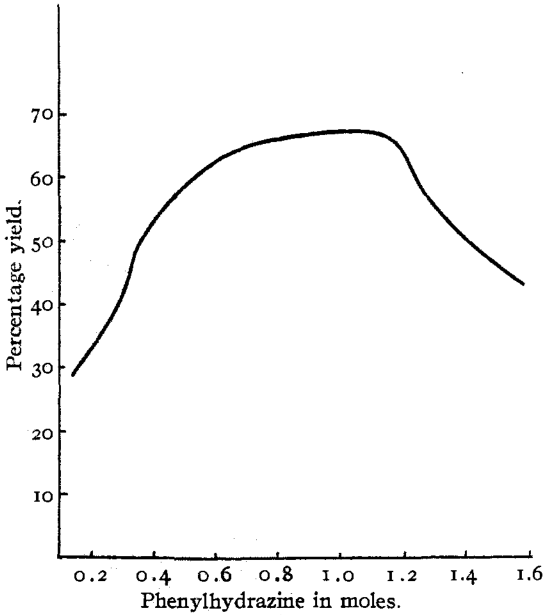

Fig. 1.—Influence of concentration of reagent upon the yield of phenylglucosazone.

Three factors may enter here: the quantity or concentration of phenylhydrazine, the acidity of the reagent, and the nature of the solvent, or rather the solubility of the osazone in the solvent which changes with the increasing percentage of acid and base present, and the resulting decrease of water. The increasing yield is undoubtedly due to the increase of concentration of the phenylhydrazine up to about 2 g. of phenylhydrazine in a volume of 20 cc. At this concentration, the phenylhydrazine acetate is about 0.92 *M* and the excess of acetic acid is 0.73 *M*. The decline in yield shown by the curve may be due either to the increased acidity or to the increased solubility of the osazone in the reagent. The first alternative was investigated by testing the acidity colorimetrically. The increase in acidity was found to be very slight. This was to be expected, however, since the presence of the acetate acts as a "buffer" to the acid. Furthermore, a test with 0.92 *M* phenylhydrazine and 3.33 *M* acid or an excess of 2.4 *M* gave a yield of 53%, which shows that any decrease in yield due to the acidity is much smaller than that observed above. No attempt was made to determine the solubility of the osazone in the mother liquor, due to the complexity and variation of the solvent. Some observations, however, indicate that the loss is due to secondary effects of the reagent. As the reagent became more concentrated, the precipitate became more and more discolored which indicates decomposition. The melting point in these cases was lower, which shows that the product is less pure. The mode of precipitation was also very different from that in more dilute solutions. The precipitate did not form so soon and came out very much slower after the first crystals began to appear.

In the light of these observations and Laves[1] results with the solubility of phenylglucosazone, it seems evident that the decrease in yield with an increase in concentration of the reagent beyond a molar solution is due to the destructive action of the reagent and the increasing solubility of the precipitate in the reagent.

Acidity.—It was thought desirable to investigate the effect of (*a*) the negative ion of the acid, (*b*) the quantity of acid used, (*c*) the actual acidity, and (*d*) the presence of a salt of a weak acid on the course of the reaction. In order to establish the first point several different acids were used. Some difficulty was experienced in obtaining suitable acids, since those of the desired strength are organic acids which were in some cases not available and in others were not sufficiently soluble. However, the following table gives a list of acids with constants which differ sufficiently for the purpose. The reactions were carried out with 0.2 g. glucose in a volume of 20 cc. and varying proportions of the acid and base. The yield is reported at the end of 1.5 hours. The constants are given at 25° for the sake of uniformity as the values at 100° in some cases are not available. The results were as given in Table I.

In addition to the results tabulated, a series of determinations were made with the concentration of the base equal to 0.92 *M* and the acetic acid varying by steps from 1.66 *M* to 4.58 *M*. In each case 0.2 g. glucose in a volume of 20 cc. was heated with the reagent for 1.5 hours. The resulting graph (Fig. 2) shows that a maximum is reached when the solution is slightly acid, *i. e.*, with a base concentration of 0.92 *M* and acid of 2.08 *M*. If the acid is increased beyond this point, the yield decreases and the solutions become badly discolored which indicates decomposition. It is found that a concentration of acid is soon reached at which there is no precipitation. The graph shows that the range of acidity within which the reaction takes place is very small. The fact that the maximum yield is reached in acid solution is probably due to the removal of NH_3 and $C_6H_5NH_2$ by the acid to form NH_4^+ and $C_6H_5NH_3^+$, respectively. These tabulated results indicate that the yield is a func-

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[1] *Loc. cit.*

TABLE I.—EFFECT OF THE ACIDITY OF THE REAGENT ON THE YIELD OF OSAZONE.

Phenylhydrazine. G.	Acid G.		Dissociation constant of acid.	Sodium acetate.	$P_{H}+$.	Yield % theory.
1.0	....		....	...	..	0.0
1.0	Phenol	1.0	1.3×10^{-10}	...	..	0.0
1.0	Boric	0.33	6.6×10^{-10}	...	..	0.0
0.4	α-Naphthol	0.4	1.0×10^{-8}	...	..	0.0
1.0	NaH_2PO_4	1.0	2.0×10^{-7}	...	..	31.2
1.0	Succinic	0.6	6.6×10^{-5}	...	..	40.0
1.0	Succinic	0.5	6.6×10^{-5}	...	..	44.13
1.0 HCl salt	....		1.0 (?)	...	..	0.0
0.5 HCl salt in 10 cc.	....		1.0 (?)	...	4.1	0.0
0.5 in 10 cc.	....		....	...	6.0	0.0
1.0 in 20 cc.	Acetic	1.0	1.8×10^{-5}	...	4.6	53.32
2.5 in 20 cc.	Acetic	2.5	1.8×10^{-5}	...	4.8	71.35
3.0 in 20 cc.	Acetic	3.0	1.8×10^{-5}	...	4.6	53.15
0.4 in 10 cc.	Acetic	0.5	1.8×10^{-5}	0.5	4.7	75.9
0.5 in 10 cc.	Acetic	0.5	1.8×10^{-5}	0.5	4.85	73.72
0.5 in 10 cc.	Acetic	0.625	1.8×10^{-5}	0.5	4.5	75.9
0.4 in 10 cc.	Acetic	0.5	1.8×10^{-5}	0.5	4.7[1]	...
0.5 in 10 cc.	Acetic	0.5	1.8×10^{-5}	0.5	4.85[1]	...
0.5 in 10 cc.	Acetic	0.625	1.8×10^{-5}	0.5	4.8[1]	...

tion of the acidity and independent of the negative ion of the acid used. The constants show the relative strength of the acids and the maximum acidity possible in each case. For values less than 10^{-7}, no precipitate is produced. Hydrochloric acid gives no precipitate when used alone, but as is well known, gives various yields when its acidity is reduced by means of a salt such as sodium acetate.

A constant of 10^{-5} seems to furnish hydrogen-ion concentration of the desired range. Any stronger acid may be used if its acidity is reduced by the use of a salt of a weak acid. This method has been very generally employed. However, the acidity is more difficult to control than where a weak acid is used.

Acetic acid is the most desirable since it is strong enough, may be regulated readily and is easy to obtain. This acid was chosen for the above reasons and the optimum acidity which was determined is shown by Fig. 2.

The hydrogen-ion concentrations were measured in several cases. The determination was made by the colorimetric method of Clark and Lubs. Since the solutions are somewhat yellow, the method consisted of using a tube of the solution behind the standard and a tube of water behind the one containing the solution and indicator. In this way compensation for the color of the solution was secured. The values are expressed as the P_{H}^{+} of Sorensen, *i. e.*, 0.0002 M $H^{+} = 2 \times 10^{-4}$, or $P_{H}^{+} = 3.7$. Differences of 0.2 were easily noticeable which makes it possible

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[1] Acidity determined after the reaction had proceeded for 1.5 hours.

to estimate to within 0.1. The standards were those of Clark and Lubs,[1] consisting of potassium acid phthalate and sodium hydroxide and were checked by means of a hydrogen electrode and static hydrogen. The solutions were shaken for half an hour in contact with hydrogen gas, before the readings were taken. A description of the apparatus and method of calculating the P_{H}^{+} for this method has been published.[2]

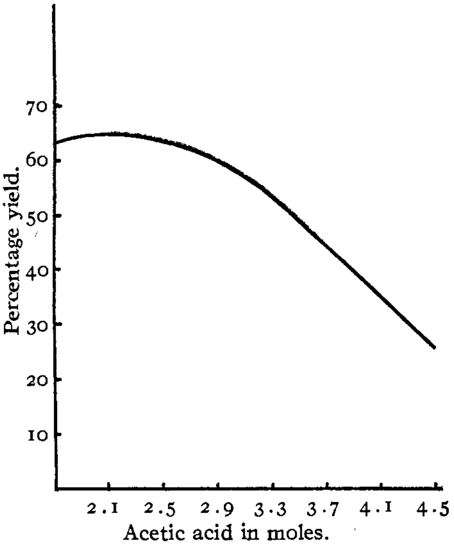

Fig. 2.—Influence of concentration of acetic acid upon yield of phenylglucosazone.

When the electrometric method was tried with solutions of the reagent directly, it was found that it did not give concordant results with these mixtures. Since the acidity does not change greatly upon heating for an hour or more, this result is probably due to the effect of the reagent on the platinum black.

The colorimetric method was much more successful, although probably not so delicate. Methyl red was used as an indicator and the results obtained by interpolation between the standards which differed by 0.2.

Table I also shows that the maximum range of acidity in which an osazone will precipitate is less than a range of P_{H}^{+} value of 2.0, *i. e.*, from $P_{H}^{+} = 4.0$ to $P_{H}^{+} = 6.0$, since at neither of these extremes is a precipitate produced. The maximum lies very close to a P_{H}^{+} value of 4.7. This corresponds pretty closely to the result obtained when different acids were used, since those acids which did not give an osazone precipitate could not give an acidity within this range.

In order to secure an excess of acid and still have the acidity low enough and constant, sodium acetate was added to the solutions. Two g. of the base, 2.5 g. of the acid, and 0.2 g. of sugar were used in each case. The results are shown in Table II.

The greatest yield is obtained with 2 *M* sodium acetate. This increase is undoubtedly due to the control of the acidity since the determination of ash in the resulting osazone showed that no important amount of sodium acetate is retained by the precipitate. The ash increases somewhat with the increased concentration but not nearly proportional to it. Potassium acetate and ammonium acetate gave results similar to those of

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[1] Clark and Lubs, *J. Bact.*, 2, 19 (1917); *J. Biol. Chem.*, 25, 479 (1916).

[2] Sherman and Thomas, This Journal, **37**, 623 (1915).

sodium acetate and so the effect is apparently due to the concentration of acetate ion.

TABLE II.—EFFECT OF SODIUM ACETATE ON THE YIELD, MELTING POINT AND TIME OF FORMATION OF THE OSAZONE.

NaAc. *M.*	Yield. % of theory.	M. p. Degrees.	Time. Minutes.
0.0	65.00	205.0	7.5
0.25	68.60	209.0	5.0
0.5	68.48	210.5	7.5
1.0	69.23	210.5	7.5
2.0	71.65	209.5	10.0
3.0	69.48	Not taken	14.0

The volume of the reacting mixture was investigated and the following results obtained:

TABLE III.—EFFECT OF THE VOLUME IN WHICH THE REACTION TAKES PLACE ON THE YIELD OF OSAZONE.

Volume. Cc.	Glucose. Mg.	Yield. % of theory.
30	200.0	65.6
30	5.0	33.0
20	200.0	76.8
20	5.0	44.5
10	200.0	84.0
10	100.0	80.4

From this it seems that 20 cc. is the best volume to use for a sample of 0.2 g. While the yield is greater in 10 cc. the amount of decomposition is also greater. However, this effect can be largely overcome by reducing the reagent also by half and thereby reducing the concentration to the original, *i. e.*, 1.0 g. phenylhydrazine, 1.25 g. glacial acetic and 0.6835 g. of crystallized sodium acetate (0.25 *M*). The greatest importance of the volume therefore, is its effect on the constant loss due to solubility of the precipitate and on the purity of the osazone.

Concentration of Sugar in the Mixture.—2.0 g. of phenylhydrazine, 2.5 g. of acetic acid and 1.367 g. of sodium acetate in a volume of 20 cc. gave, when heated for 1.5 hours, the results shown in Cols. II and III under yield in Table IV.

This series was repeated with 0.5 g. phenylhydrazine, 0.5 g. acetic acid, and 0.5 g. sodium acetate in a volume of 10 cc. heated for 2 hours, with the results shown in Col. IV.

There is a constant loss due to the solubility of the osazone in the reagent which will appear as a decreasing yield due to the fact that it is a larger percentage of the smaller sample. From the complex nature of the solvent, it is difficult to determine the exact value of this solubility but it is less than 10 mg. to 10 cc. of the reagent, since 5 mg. of glucose has a theoretical yield of 10 mg. Assuming that this is the only loss from 5 mg. of sugar, the loss is 6.5 mg.

TABLE IV.—EFFECT OF THE CONCENTRATION OF GLUCOSE ON THE YIELD OF OSAZONE.

I. Glucose. Mg.	Yield. % of theory.		
	Higher conc. of reagent.		Lower conc. of reagent.
	II.	III.	IV.
200	76.95	76.7	73.72
150	75.95	...	72.43
100	71.65	71.8	70.55
50	65.6	...	68.20
20	...	...	59.5
10	48.50	49.00[1]	48.0
5	45.00	44.00[1]	35.0
2	Slight on cooling		

Wash Water.—A determination was made with 1.0 g. phenylhydrazine, 1.25 g. acetic acid, 0.6835 g. sodium acetate (0.25 *M*) in a volume of 10 cc. The whole was heated 2 hours, cooled to 60° and filtered. Wash water at room temperature was added in 10 cc. portions, collected separately and weighed, evaporated to dryness and the residue weighed, with the results shown in Table V.

TABLE V.—SOLIDS REMOVED BY SUCCESSIVE WASHINGS.

No.	Volume. Cc.	Solids. G.		
		I.	II.	III.
1	10	0.0520	0.0532	0.0566
2	10	0.0053	0.0258	0.0182
3	10	0.0061	0.0070	0.0058
4	10	0.0025	0.0029	0.0026
5	10	..	0.0016	0.0017
6	10	..	..	0.0011
7	10	..	..	0.0004

The washing is practically complete after the fifth 10 cc. portion of water has been used. Less than 2 mg. was removed by the fifth portion and that was probably mostly osazone, as the solution was distinctly yellow. No attempt was made to analyze this residue, however, since it decomposed (became discolored) during the evaporation of the water.

Duration of the Reaction.—The time necessary for the maximum yield of osazone has been variously reported. Some of the determinations made in the course of this investigation gave the results shown in Table VI.

TABLE VI.—EFFECT OF THE DURATION OF HEATING ON THE YIELD OF OSAZONE.

Time. Hours.	Yield. % of theory.
1.0	56.9
1.5	65.9
2.0	76.5
2.5	77.0
3.0	81.0
4.0	Decomposed

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[1] Molar sodium acetate was used in checks.

The reaction is not complete in less than 3 hours. Beyond this time it is impossible to say because of the decomposition which takes place under prolonged heating. From these results, it is most desirable to heat for 2 hours since that time produces the greatest yield of a relatively pure product. The speed of the reaction like the yield is a function of the concentration of reagent used and of the acidity. In no case has the reaction been found nearly complete in less than 2 hours and in no case has a greater time seemed desirable. While a less acid solution produces less decomposition of the osazone, it also results in a slower reaction.

Application to the Detection of Single and Mixed Sugars.—The conditions which produce the largest yield with a sample of 0.2 g. are not those best adapted to detecting minute amounts of glucose. This is probably due to the fact that the osazone is more soluble in the phenylhydrazine-acetic acid mixture than it is in water. While more osazone may be formed in the case of the more concentrated reagent it does not separate out of the solution so readily. The most important results are reported in Tables VII, VIII, IX, X and XI.

TABLE VII.—EFFECT OF CONCENTRATION OF THE REAGENT ON THE TIME OF PRECIPITATION WHEN GLUCOSE IS IN 0.05% SOLUTION AND VOLUME EQUALS 10 CC.

Phenyl-hydrazine.	Acetic acid. G.	Sodium acetate. G.	Time.
0.6	0.4	0.5	None-hot
0.8	0.5	0.5	In 4 hrs.
0.8	0.8	0.5	None in 2 hrs.
0.6	0.6	0.5	70 min.
0.4	0.4	0.2	63 min.
0.4	0.5	0.2	50 min.
0.4	0.5	0.5	47 min.
0.5	0.5	0.5	54 min.
0.5	0.625	0.5	82 min.
0.4[1]	0.5	0.5	None in 2 hrs. or on cooling
0.5[1]	0.5	0.5	

In Table VII the results are tabulated from the experiments made for the purpose of finding the conditions necessary to determine a minimum quantity of glucose. From these and other experiments it appears that with 0.005 g. of glucose, 0.4 g. phenylhydrazine, 0.5 g. acetic acid, and 0.5 g. sodium acetate produce a precipitate in the least time. Those conditions in which the base predominates are distinctly inferior and so are discarded in future experiments. It may be noticed also that an increase in the concentration of the reagent lessens the delicacy.

In Table VIII the 3 most likely sets of conditions are compared and the limits given in each case. Here it is seen that the conditions given in the preceding paragraph are the best, while an equal weight (0.5 g.) of each of the 3 reagents is only slightly inferior. With the best condi-

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[1] Volume was 4 cc. in these 2 tests only.

TABLE VIII.—EFFECT OF THE CONCENTRATION OF GLUCOSE ON THE TIME OF PRECIPITATION WHEN A VOLUME OF 10 CC. CONTAINING 0.5 G. SODIUM ACETATE WAS USED IN EACH CASE.

Dextrose. G.	Time.
Using 0.5 g. Phenylhydrazine and 0.5 g. Acetic Acid	
0.200	6 min.
0.100	5 min.
0.010	19 min.
0.005	54 min.
0.002	None in 3 hrs.—distinct when cool
0.001	None
Using 0.4 g. Phenylhydrazine and 0.5 g. Acetic Acid.	
0.200	5 min.
0.100	5 min.
0.010	14 min.
0.005	47 min.
0.002	None in 3 hrs.—distinct when cool
0.001	Ppt. on cooling after 3 hrs. heating
Using 0.5 g. Phenylhydrazine and 0.625 g. Acetic Acid.	
0.100	6 min.
0.010	27 min.
0.005	82 min.
0.002	None in 3 hrs.—distinct when cool
0.001	None

TABLE IX.—CONDITIONS OF PRECIPITATION FROM SOLUTIONS OF SUCROSE, MALTOSE AND LACTOSE WHEN A VOLUME OF 20 CC. WAS USED WITH 1 G. OF PHENYLHYDRAZINE AND 10 CC. IN ALL OTHER CASES.

Phenylhydrazine. G.	Acetic acid. G.	Sodium acetate. G.	Time. Min.	Yield. % of theory.
		Using 0.200 g. Sucrose.[1]		
0.4	0.5	0.5	30	..
0.5	0.5	0.5	33	..
0.5	0.625	0.5	29	..
1.0	0.8	1.367	85	..
1.0	1.25	0.6835	60	..
1.0	1.25	None	45	..
1.0	1.00	1.367	80	..
1.0	1.00	None	48	..
1.0	1.00	1.367	70	..
1.0	0.6	1.5	100	..
		Using 0.100 g. Sucrose.[1]		
1.0	0.6	1.5	130	..
		Using 0.200 g. Maltose.[2]		
0.4	0.5	0.5	...	41.8
0.5	0.5	0.5	...	40.6
		Using 0.200 g. Lactose.[3]		
0.4	0.5	0.5	...	54.7
0.5	0.5	0.5	...	54.6

[1] Precipitation begins at 100°; [2] at 63°; [3] at 65° in the first and 64° in the second experiment.

Table X.—Effect of Maltose on the Precipitation of Glucosazone When a Volume of 10 Cc. is Used.

Phenyl-hydrazine. G.	Acetic acid. G.	Sodium acetate. G.	Maltose. G.	Glucose. G.	Time. Min.	Yield in 2 hrs.
0.5	0.5	0.5	0.050	0.150	5.5	80.26 at 70°
0.5	0.5	0.5	0.100	0.100	6.5	86.35 at 70°
0.5	0.5	0.5	0.150	0.050	8.0	70.40 at 70°
0.5	0.5	0.5	0.180	0.020	29.0	54.25 at 70°
0.5	0.5	0.5	0.190	0.010	None-hot	5.0 at 70°
0.5	0.5	0.5	0.195	0.005	None-hot	....
0.4	0.5	0.5	0.180	0.020	40.0	51.25
0.4	0.5	0.5	0.190	0.010	None in 4 hrs.	....
0.4	0.5	0.5	0.190	0.010	None in 4 hrs.	....
0.5	0.5	0.2	0.190	0.010	None in 4 hrs.	....
0.5	0.625	0.5	0.190	0.010	None in 4 hrs.	....
0.5	0.5	0.5	0.150	0.050	10.0	....
0.5	0.5	0.5	0.180	0.020	35.0	....
0.5	0.5	0.5	0.180	0.020	34.0	40.0[1]

tions it is possible to detect 5 mg. of glucose in 10 cc. of solution since a distinct precipitate is formed. If the solution is cooled, 1 mg. gives a distinct precipitate. If the original solution is neutral and comparatively free from other organic matter this test will show 1 part of glucose in 10,000 of water or 1 mg. in a 0.01% solution. At this dilution the precipitate is sufficiently copious, so that there is no question about its presence.

Since it is sometimes necessary to determine glucose in the presence of sucrose, the experiments reported in Table IX were made. As is well known, sucrose forms a glucosazone because it is hydrolyzed by the acid character of the reagent mixture. It is possible to reduce the acidity until sucrose will not be sufficiently hydrolyzed to give a precipitate on being heated for over an hour when 0.2 g. is present. These same conditions will give a precipitate with glucose in a few minutes. Table IX also shows the effect of sucrose when it is in a mixture. With small amounts of sucrose, the time required for the precipitate to form is not greatly changed, while with 2.5% glucose the time of formation of the precipitate shows that the sample is not pure sucrose.

Some cooling below 100° is unavoidable during filtration and so determinations were made with lactose and maltose to find at what temperature they begin to give a precipitate. The results show that no danger from this source is met with, so long as the temperature is kept above 70°, since maltosazone and lactosazone do not begin to crystallize until the solution is cooled to about 65°. If the filtration is carried out promptly the solution may be kept much above this temperature.

The effect of these sugars on the glucosazone reaction (maltose in Table X, lactose in Table XI) is to retard it, as has been shown by former

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[1] Cooled to room temperature and reheated.

Table XI.[1]—Effect of Lactose, Sucrose, Starch and Dextrin on the Precipitation of Glucosazone.

Lactose. G.	Glucose. G.	Time. Min.	Yield in 2 hrs.
0.150	0.050	14	....
0.180	0.020	45	....
0.180	0.020	46	....
0.000	0.020	18	....
0.050	0.150	5	72.2%
0.100	0.100	7.5	70.35%
0.150	0.050	13	63.90
0.180	0.020	39	22.75
0.190	0.010	...	1.0(?)[2]
Sucrose.			
0.050	0.150	4.5	78.73%
0.100	0.100	5.5	81.55
0.150	0.050	7.0	95.8
0.180	0.020	13.0	150.5
0.190	0.010	23.0	245.0
0.195	0.005	28.0	409.0
Starch.			
0.050	0.150	5.0	71.30%
0.100	0.100	6.0	69.35
0.150	0.050	8.0	67.00
0.180	0.020	16.0	58.50
0.190	0.010	36.0	47.0
0.195	0.005	120.0?	14.0
Dextrin.			
0.050	0.150	5	67.6%
0.100	0.100	7	(45.5)
0.150	0.050	13	66.5
0.180	0.020	28	59.2
0.190	0.010	...	71.5
0.195	0.005	...	70.0

workers. With 90% maltose and 10% glucose the time is retarded from 15.5 min. to 28 min. With 95% maltose and 5% glucose, no osazone is formed unless the solution is cooled. Some of this precipitate does not redissolve on reheating. Since all maltosazone and lactosazone dissolve on heating this residue must be glucosazone and so if the sample is a mixture of maltose and glucose, 5% glucose is the minimum limit of this reaction. Heating for a greater length of time does not give a precipitate with the 5% glucose in the presence of 95% of either maltose or lactose.

The effect of lactose is seen to be greater than that of maltose, as was reported by Sherman and Williams.[3] While 90% maltose retards the

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[1] Reagent is 0.5 g. phenylhydrazine, 0.5 HAc, 0.5 g. NaAc in a volume of 10 cc. None were discolored with starch present.

[2] Cooled to room temperature and reheated.

[3] *Loc. cit.*

precipitation to 29 min. the 90% lactose retards it to 45 min. With lactose the minimum limit of the reaction is the same, under the conditions here used, as with maltose, *i. e.*, 10% hot, and 5% by cooling and reheating, was the smallest proportion of glucose in lactose which gave a satisfactory test.

As the concentration of the reagent increases, the time required for precipitation of the osazone increases. As has been suggested at an earlier point, this is due to the fact that the osazone is more soluble in the reagent than it is in water and that it crystallizes out more slowly. In view of this, the retarding effect of disaccharides is apparently due to the presence of their osazones and not, as has sometimes been supposed, to the reduced concentration of the reagent due to its removal to form the disaccharide osazones. This view is further supported by the fact that the retarding effects of equivalent quantities of maltose and lactose are not the same.

The effect of starch is peculiar. The time of precipitation is only slightly less. With less than 50% starch it may be neglected altogether. The precipitates were less discolored than when no starch was used, and the yield was very little affected.

The effect of dextrin is very similar to that of maltose. This is especially noteworthy from an analytical standpoint, since both are likely to be present in the same solution.

A knowledge of the effects of the various factors above recorded makes it possible to interpret the results of an osazone reaction in a wider variety of cases than has been possible heretofore. In the interpretation of a given result, the yield, the melting point of the osazone, and the time required for the formation of a precipitate should, in general, be employed. There are cases, however, where merely the formation of a precipitate is conclusive. In order to avoid erroneous conclusions, it should be borne in mind that pentoses as well as other hexoses affect the results obtained.

The reaction was applied to one analytical problem. Starch had been hydrolyzed by an enzyme (taka-diastase) and it was desirable to know whether or not glucose was formed by the reaction. 8 cc. of the solution was added to 0.5 g. of phenylhydrazine, 0.5 g. acetic acid and 0.5 g. sodium acetate. The mixture was heated for 2 hours, filtered hot, and washed with 40 cc. (4 × 10 cc.) of distilled water at room temperature. The time was taken in each case and the yield in 3 of them.

TABLE XII.—GLUCOSAZONE PRECIPITATION FROM UNKNOWN SOLUTIONS.

Solution No.	Time. Min.	Yield. Mg.	Glucose. Mg.
3	12	15.0	12.7
4	31	13.3	11.6
7	10	..	..
8	5(?)	7.5	7.6(?)

From these results it is obvious that all of the samples contain glucose and some idea is obtained of the amount of it. From Table IV it is seen that if 4.0 mg. are added to the weight of osazone formed in a given case, the yield is then about 75% of the theoretical, or 1.5 times the weight of glucose. From this it is possible to calculate arithmetically the quantity of glucose, *i. e.*, $\frac{\text{mg. of osazone} + 4.0 \text{ mg.}}{1.5} = \text{mg. of glucose.}$ At this dilution, however, the percentage yield is not very reliable, since small losses affect the result greatly. It does serve to establish a minimum quantity of glucose, since neither maltose, starch, nor dextrin yields any precipitate when treated in a similar manner. If the time is considered it will also serve to establish a minimum glucose content. The precipitate from solution No. 4, Table XII, came down in 31 minutes. With pure glucose this would mean about 10 mg. of glucose, but with 75 mg. of maltose present the precipitation is retarded and so the quantity of glucose is much in excess of 10 mg.

Conclusions.

1. The correct melting point of phenylglucosazone is 208.0° (corr.) regardless of the method of purification.

2. There is a concentration of phenylhydrazine which produces a maximum yield of glucosazone; any increase in the concentration above this results in a rapidly decreasing yield, probably due to the increased solubility of the osazone in the reagent.

3. The efficiency of the reagent depends upon the acidity and is independent of the negative ion of the acid used.

4. The range of acidity in which the reaction takes place is rather slight, between $P_{\text{H}}^+ = 4.0$ and 6.0. The maximum lies close to 4.7 and is best secured with acetic acid and sodium acetate. Very slight change in acidity takes place during the reaction.

5. Within fairly wide limits a variation of the size of the sugar sample used has very little effect on the percentage yield of osazone beyond the effect due to the constant loss which results from the solubility of the osazone.

6. With such quantities of sugar and reagents as were here used, 4 10 cc. portions of water at room temperature wash the precipitate sufficiently for analytical purposes.

7. While maltose and dextrin retard the formation of the precipitate, lactose retards it to a greater extent and starch has very little effect.

8. The limit of the delicacy of the test for glucose has been extended.

9. The tabulated results of experiments with both pure sugars and mixtures extend the analytical application of the reaction.

10. The reaction continues at least three hours.

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