The Action of Enzymes on Invertase.—Wroblewski[1] showed that invertase was not destroyed by the action of trypsin, and Mathews and Glenn found that the gum was not digested by diastase. Similar results were obtained in this laboratory with ptyalin and pancreatic amylase. They were allowed to stand in contact with the gum from invertase over 12 hours, and at the end of that time no reduction with Fehling's solution could be noticed. Castor bean lipase also gave negative results.

The authors wish to take this opportunity to express their thanks and appreciation to the Jacob Ruppert Brewery, New York City, for furnishing such ample amounts of pressed yeast.

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THE PICRATE COLORIMETRIC METHOD FOR THE ESTIMATION OF CARBOHYDRATES.

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The quantitative estimation of sugars and other carbohydrates involves the use of the polarimeter or the oxidizing power of copper solutions; as yet no accurate application of colorimetric methods[2] has been made to carbohydrates.

The method herein described depends upon the formation of a red-to-brown color when solutions of sugars are heated with *sodium carbonate solutions of picric acid.* These colors were first described by Braun;[3] other observers were Jaffé,[4] Johnson[5] and Chapman.[6] All these investi-

[1] *J. prakt. Chem.*, **64**, 1 (1901).

[2] Dubrunfaut devised a method for estimating glucose by comparing the color developed by treating sugar solutions with alkali. Heller (*Archiv.*, **1**, 212; **4**, 310; *Z. anal. Chem.*, **28**, 650; *Deut. med. Wochschr.*, **1888**, 451) made modifications of this method. Neitzel (*Chem. Ztg.*, **1894**, Rep. 93; *Z. Rübenzucker-ind.*, **1894**, 221) used a modification of Molisch reagent (*Monatsh.*, **7**, 198). Johnson (*Z. anal. Chem.*, **23**, 111; *Brit. Med. J.*, **1883**, 504; *Pharm. J. Trans.*, **54**, 24; *Deut. med. Wochschr.*, **1888**, 451, 479; *Pharm. Prax.*, 1880, 1, 103) used picric acid and potassium hydroxide. Autenreith and workers (*Münch. med. Wochsch.*, **57**, 1780; **58**, 899; **59**, 689) oxidized by Bang's solution (*Biochem. Z.*, **2**, 271; **32**, 443) and estimated colorimetrically the unchanged copper. Järvinsen (*Z. anal. Chem.*, **52**, 14) makes use of Almen-Nylander's reagent (*Ibid.*, **9**, 494; *Z. physiol. Chem.*, **50**, 36; *Biochem. Z.*, **16**, 489). For other applications of colorimetry to carbohydrates see Neitzel, *Z. Spiritusind.*, **20**, 163; Ruini, *Gazz. chim. ital.*, **31**, 445; Lyons, *Pharm. Rev.*, **20**, 155; Dennstedt and Voigtländer, *Forschungsb. ü. Lebensmittel*, **2**, 173; Ambuhl, *Chem. Ztg.*, **19**, 1508.

[3] *Z. Anal. Chem.*, **4**, 185; *Chem. Zentr.*, **1866**, 219; **1874**, 825; *J. prakt. Chem.*, **96**, 412. Braun mentions that glucose, fructose and lactose give these colors.

[4] *Z. physiol. Chem.*, **10**, 391.

[5] *Pharm. J. and Trans.*, **54**, 24.

[6] *Analyst*, **34**, 475.

gators used caustic alkalies instead of sodium carbonate, hence the colors obtained resulted not only from the reduction of picric acid by the sugars, but also from the caramelization of sugars by the alkali.[1] This latter effect is entirely avoided in the use of sodium carbonate.

Braun states that picramic acid is formed in these reactions. In the reduction of alkaline solutions of picric acid by creatinine, Chapman[2] holds that three stages of reduction are involved, *i. e.*, one, two, or three nitro groups are transformed to amino groups. We have observed that, whereas aldehydes, ketones, keto acids, creatinine, etc., give with alkaline solutions of picric acid a *red* color which usually is dissipated by an excess of the substance, the picrate product of reduction by sugars varies with increasing concentrations through yellow, red, brown to black, and is permanent, except toward light, in the presence of an excess of the substance oxidized.

Since the color[3] does not match closely with the colors of solutions of ferric acetate,[4] potassium bichromate, or *o*-nitrophenolate, we have adopted *the color of the sugar-picrate solution itself*, as the standard of color and thus, of course, obtain absolute identity of color.

Molecular equivalents of any available pure sugar, as sucrose, lactose, or glucose, may be used to make up the standard color solution. Since sucrose is anhydrous and can be obtained both cheaply and in the pure form, it is the best. However, since in the determination of the respective sugars, as for instance lactose and glucose, a reference to a particular sample of sugar may be desired, such sample may be made the standard with equal ease.

For convenience of calculation the solution used is best prepared so as to be read directly as *anhydrous monosaccharide*. Thus, for instance, to be equivalent to one gram of anhydrous glucose, the following quantities of other sugars[5] must be used:

[1] *Chem. Zentr.*, **1847**, 623; *Lancet*, **25**, Sept., 1844; *Chem. Ztg.*, **1901**, *Rep.*, 209; *Münch. med. Wochschr.*, **1906**, 1309.

[2] *Chem. News*, 100, 175; *Brit. Med. J.*, Dec. 12, 1908.

[3] When an excess of glucose was heated with the picrate solution, afterwards cooled and then treated with dilute sulfuric acid, an amorphous precipitate was formed. It was filtered, washed with water, redissolved in alkali and reprecipitated by sulfuric acid. After washing first with water and then with alcohol, it was dried in a vacuum desiccator over sulfuric acid. Thus prepared, the substance was brown-black and did not melt at 270°. Since 4,6-dinitro-2-aminophenol melts at 169° and 2,6-dinitro-4-aminophenol melts at 170°, the substance cannot be either of these. Since the alkaline salt of triaminophenol gives, with ferric chloride, a violet color, while this substance does not, it cannot be the triamino compound. With hot water the substance yields a difficultly soluble black residue and an easily soluble brown solute, hence it must be a mixture. Its composition will be investigated later.

[4] Johnson, *Pharm. J. and Trans.*, **54**, 24.

[5] For the preparation of pure sugars from commercial samples, see the following: *Sucrose*, THIS JOURNAL, **23**, 61; *U. S. Bur. Chem. Bull.*, **73**, 58; *Glucose*, Munson and

Sucrose	0.950
Glucose monohydrate	1.100
Lactose monohydrate	1.000
Lactose (anhydrous)	0.950
Lactose ($^1/_2$ mol H_2O)[1]	0.975

The picric acid solution is so prepared that it contains two grams of the acid and four grams of anhydrous sodium carbonate per liter; thus, in the color standard, twice as much picric acid as sugar is used. An excess of picric acid is necessary not only to insure complete oxidation of the sugar, but, as observed by Chapman, to prevent the formation of colorless reduction-products of the picric acid. On the other hand, too large an excess is to be avoided since it has an effect on the colorimetric reading.

Sodium carbonate, instead of sodium hydroxide,[2] is used because it avoids both the hydrolytic and the decomposing effect of the latter. In using the same concentration of different carbohydrates and treating under identical conditions, we have found that: (1) sodium carbonate solutions of picric acid at room temperatures give no increased color with carbohydrates, even after weeks' standing, except in the case of fructose, which slowly darkens; (2) *on boiling*, all sugars except sucrose and raffinose develop color immediately, with sodium carbonate solutions of picric acid; (3) sodium hydroxide solutions of picric acid, *at room temperature*, slowly develope color with all sugars and many other carbohydrates.

Preparation of Solutions.

(A) *The picrate solution* is prepared as described above.

(B) *The standard color solution* is prepared as follows: Dissolve 0.95 gram of pure cane sugar in about 100 cc. of water contained in a 500 cc. casserole and add 5 cc. of concentrated hydrochloric acid.[3] Heat on the water bath 15 minutes, dilute and cool, add an excess of sodium carbonate solution and 100 cc. of the picrate solution. Boil for 5 minutes after the color begins to develop and dilute to one liter. The color of this solution[4]

Walker, THIS JOURNAL, **28**, 667; *Lactose*, Lippman, *Die Chemie der Zuckerarten*, 3 Auflage, 1526; Walker, THIS JOURNAL, **29**, 541; *Maltose*, Walker, *Ibid.*, **29**, 542; also see *Ibid.*, **34**, 326.

[1] See THIS JOURNAL, **29**, 54.

[2] For the effect of alkalies on sugars, see especially: Benedict, *J. biol. Chem.*, **3**, 101; **5**, 485; Kendall, THIS JOURNAL, **34**, 317; McLean, *Biochem. J.*, **2**, 156; Jolle, *Z. Nahr. Genussm*, **20**, 631.

[3] For the hydrolysis of cane sugar, see Wiley's Agricultural Analysis, Vol. III, 105–107; Bornträger and Samelson, *Z. angew. Chem.*, **1892**, 334; **1893**, 690; **1894**, 267, 351; Munson and Walker, THIS JOURNAL, **28**, 667.

[4] This 0.1% solution of reduced picrate possesses nearly the same color and intensity of color as a 3% solution of potassium dichromate; it is nearly 30 times as intensely colored as the dichromate.

is equivalent to that produced by 1 gram (0.1%) of anhydrous monosaccharide per liter. This solution is too concentrated for use in the colorimeter; 100 cc. of it should be diluted to one liter (0.01%). For the "bottle standards" described below, however, it is the most convenient starting solution.

General Method of Manipulation.

Carefully weigh out approximately one gram of the carbohydrate or mixture of the same containing no interfering[1] substance. The monosaccharides, lactose and maltose react directly when heated with the picrate solution; hydrolyze sucrose and raffinose by adding 5 cc. of concentrated hydrochloric acid and heating for 15 minutes on the water bath. Neutralize with sodium carbonate solution and make up to one liter in a volumetric flask. Run exactly 10 cc. of this solution into a 100 cc. volumetric flask, add 10 cc. of the picrate solution and heat on the sand bath so that the solution boils for 5–10 minutes. Cool and add water to the 100 cc. mark. Place this solution in a standard colorimeter cylinder and adjust its depth to any depth of the standard color solution contained in the other colorimeter cylinder.

Calculation.—Divide the reading of depth of solution in the color-standard cylinder by the reading of depth of solution in the other cylinder and multiply by the weight of substance taken and the quotient[2] is *grams of anhydrous monosaccharide per liter*. The following table gives the factors necessary to convert to the other sugars or their hydrated forms:

Carbohydrate.	Formula.	Mol. wt.	Factor.
Rhamnose	$C_6H_{12}O_5$	164.096	0.9111
Glucose	$C_6H_{12}O_6.H_2O$	198.112	1.1000
Glucose	$C_6H_{12}O_6$	180.096	1.0000
Galactose	$C_6H_{12}O_6$	180.096	1.0000
Mannose	$C_6H_{12}O_6$	180.096	1.0000
Fructose	$C_6H_{12}O_6$	180.096	1.0000
Maltose	$C_{12}H_{22}O_{11}.H_2O$	360.192	1.0000
Maltose	$C_{12}H_{22}O_{11}$	342.176	0.9500
Lactose	$C_{12}H_{22}O_{11}.H_2O$	360.192	1.0000
Lactose	$C_{12}H_{22}O_{11}$	342.176	0.9500
Sucrose	$C_{12}H_{22}O_{11}$	342.176	0.9500
Raffinose	$C_{18}H_{32}O_{16}.5H_2O$	594.336	1.1000
Raffinose	$C_{18}H_{32}O_{16}$	540.288	1.0000

[1] The color of certain solutions, as of syrups, molasses, urine, etc., interfere; also polyphenols, aldehydes, ketones, purine bases, etc., will reduce the alkaline picrate solution. A systematic survey of compounds treated with alkaline picrate solution will be given later.

[2] It must be remembered that both the color standard and the unknown were first dissolved in one liter, then diluted to one-tenth concentration. If other dilutions of the color standard are used, the decimal point must, of course, be altered accordingly. In case of a low percentage of sugar in the unknown and when about 1 gram is used, it will be advantageous to dilute only to one-liter.

Example.—When 1.0200 grams of a sample containing lactose and some indifferent substance was treated in the above-described manner, a reading of 25.5 mm. in the colorimeter cylinder was exactly matched by 20 mm. depth of the standard color solution contained in the other cylinder. That is 20/25.5 of 1.0200 is equal to 80% of anhydrous monosaccharide and this multiplied by 0.95 (see table) gives 76% of anhydrous lactose in the original sample.

Colorimeters and a Proposed Substitute.—Of course, any of the types of colorimeters may be used but Duboscq's is especially to be recommended. In the absence of a colorimeter, bottles of clear glass and of the same size and shape may be used. Square bottles of about 40 mm. thickness and 60 cc. capacity are especially convenient, though nearly any uniformly shaped and sized bottle may be used.

From the standard color solution prepared as described above, solutions of the following percentage-concentrations are prepared:

0.1	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02
0.01	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002
0.001	0.0009	0.0008	0.0007	0.0006	0.0005	0.0004	0.0003	0.0002
0.0001	0.00009	0.00008	0.00007	0.00006	0.00005	0.00004	0.00003	0.00002

Since concentrations above 0.05% in the 40 mm. bottles are nearly opaque and below 0.0005% are nearly the same in color as the picrate solution, only "bottle standards" from 0.03% to 0.0005% need be prepared, that is, only seventeen in all, a task that need not consume more than two hours of time.

To use these "bottle standards," the carbohydrate of unknown concentration is treated as described above and its colored solution is placed in a bottle of the same size and shape as the color standard bottles, a little of the solution being first used to rinse out the bottle. A comparison is then made with the color standards, the bottles being placed on white paper and viewed from some distance. Viewing the bottles with nearly closed eyes and from distances of six or more feet is recommended, not only as involving no eye-strain but as enhancing the sensitiveness of reading. Attention is called to the fact that when the colors of the unknown and the standard appear identical if viewed transversely, a viewing of the same longitudinally will often show a difference. With a little practice the matching of the unknown with the standard may be made in a few moments.

Theoretically, in viewing two or three bottles *in tandem* and alongside of the bottle containing the unknown, any decimal of concentration from 0.05 down to 0.0005 may be read. In practice, to determine the concentration of the unknown, only one bottle standard or two *in tandem* are

matched against the unknown. This results from the following conditions: (1) a finer reading is unnecessary for most analytical purposes; the digits indicating the concentrations give readings as parts per 10,000, 100,000 or 1,000,000; (2) in accordance with Weber's law of sensation, the eye is not sensitive[1] to all differences of concentration, especially in the upper part of a series.

Durability of the Color Standards.—Since the color standards are not strictly proof to sunlight they should be kept in the dark when not in use, However, the following data show that their rate of deterioration is slow. Series (A) was freshly prepared; series (B) was kept for twenty days in summer sunlight.

A.	B.	A.	B.	A.	B.	A.	B.
0.070	0.080	0.020	0.033	0.008	0.013	0.0009	0.0015
0.060	0.070	0.018	0.030	0.007	0.012	0.0008	0.0012
0.050	0.060	0.016	0.025	0.006	0.010	0.0007	0.0009
0.045	0.060	0.015	0.023	0.005	0.008	0.0006	0.0008
0.040	0.055	0.014	0.020	0.004	0.006	0.0005	0.0006
0.035	0.050	0.012	0.018	0.003	0.0044	0.0004	0.0005
0.030	0.045	0.010	0.016	0.002	0.0030	0.0003	0.0004
0.025	0.035	0.009	0.015	0.001	0.0016	0.0002	0.0002

It will be observed that to match the respective concentrations of (A), invariably greater indicated concentrations of (B) were necessary, hence, with very old standards the analytical data will be too high. It is recommended that a stock solution of the color solution (0.1%) be prepared and preserved in the dark; from it new "bottle standards" may be prepared when necessary.

Colorimetric Readings by Artificial Light.—As is well known, the judging of shades of color by artificial light leads to error, especially in the case of yellows. Reds and browns are little liable to such error, hence the upper part of the color-standard series (the 0.05% to 0.0005% part recommended above) is adapted to use in artificial light. This is true for the reasons not only that the reds and browns are persistent but that the yellow of the excess of picrate solution, theoretically, at least, a disturbing influence, is reduced to *nil* in such light.

Accuracy of the Method.—Equal concentrations (0.1%) of the following carbohydrates were prepared and preserved with toluene: the polysaccharides were finely divided, suspended in water and shaken thoroughly before being measured out. Each was treated with the picrate solution in the manner described above and finally diluted to 0.01% concentration.

[1] For discussion of variable sensitiveness in colorimetry see Horn and Blake, *Am. Chem. J.*, **35**, 253; **36**, 195, 516.

Carbohydrate.	Mols H_2O.	Standard.	Calc. conc.	% error.
Glucose	..	0.01	0.01	..
Fructose	..	0.01	0.01	..
Galactose	..	0.01	0.01	..
Mannose	..	0.01	0.01	..
Rhamnose	1	0.01	0.0101	1.00
Maltose	1	0.0094	0.009895	1.05
Lactose	1	0.0095	0.01	..
Sucrose	..	....	....	..
Sucrose (hydrolyzed)	..	0.0105	0.009974	0.26
Raffinose	5	....	....	..
Inulin	..	....	....	..
Dextrin	..	0.002	?	?

Lactose in Milk.—Measure out 10 cc. of milk and run it into a 250 cc. volumetric flask with 50–100 cc. of water and 1 cc. of 50% sulfuric acid. Heat on the sand bath until flocks of casein separate or until the mixture boils. Cool and add water to the 250 cc. mark. Pour upon a dry filter and, by means of a graduated pipet, take 6.25 cc. of the filtrate and run it into a 250 cc. volumetric flask. Add 2–3 volumes of water, an excess of sodium carbonate solution, and 10 cc. of the picrate solution. Heat on the sand bath five minutes after the color begins to develope. Cool and dilute to the 250 cc. mark. Estimate the color by the colorimeter or by means of the bottle standards. For the latter, rinse the bottle with some of the solution, then fill and compare with bottle standards. Multiply the percentage marked on the bottle standard by 1000 as the percentage of lactose in the milk.

Advantages of the Method.—A speedy and accurate *colorimetric* method for most carbohydrates is introduced.

Only one oxidizing solution, the picrate solution, is necessary and it is cheap, stable, easily prepared and need not be strictly quantitative.

The oxidizing solution is alkaline with sodium carbonate, avoiding secondary reactions on sugars produced by caustic alkali.

With the use of the bottle standards, the method is not only inexpensive but is easily within the reach of all working chemists.

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[CONTRIBUTIONS FROM THE CHEMICAL LABORATORY OF THE UNIVERISTY OF WASHINGTON.]

A COMPARISON OF VARIOUS PRESERVATIVES OF URINE.

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In certain studies on normal urines, incurring the collection and keeping of hundreds of liters, the necessity of using a non-volatile preservative compelled us to test a number of reagents to determine their preservative power.