very soluble in water, soluble in alcohol, and sparingly soluble in acetone and petroleum ether. On treating an aqueous solution of it with acetic acid, crystals of the mono-iodo-saligenin, melting at 138° are obtained.

Summary.

1. A method has been developed of treating saligenin in acetone solution with alcoholic potash by which practically theoretical yields of the potassium saligenate were obtained. This method was also used successfully in the preparation of the potassium salts of monobromo- and mono-iodo-saligenin.

By the reaction of potassium saligenate on ethyl iodide, the ethyl ether of saligenin identical with ethyl ether of saligenin prepared by Bötsch was prepared. In an analogous manner the propyl, *n*-butyl, *iso*-amyl, and benzyl ethers of saligenin were made.

3. The mono-acetate and the monobenzoate of saligenin were prepared from potassium saligenate and acetic anhydride and benzoyl chloride, respectively. The dibenzoate of saligenin was made by the benzoylation of saligenin in pyridine in the presence of calcium carbonate and with a slight excess of the benzoyl chloride.

4. The method of Auwers and Büttner for preparation of monobromo-saligenin by the bromination of saligenin in aqueous solution was found to give the best results.

5. For the preparation of mono-iodo-saligenin the best method found was the treatment of saligenin dissolved in water with an aqueous potassium iodide solution of iodine.

MINNEAPOLIS, MINNESOTA.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF COLUMBIA UNIVERSITY, No. 359.]

SOME ERRORS IN THE STUDY OF INVERTASE ACTION.

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Received April 28, 1921.

It was pointed out by Nelson and Vosburgh[2] that a source of error about which little was known existed in the measurement of the velocity of hydrolysis of sucrose by invertase. The trouble was thought to consist in a change in activity which took place when invertase solutions were diluted, and the magnitude of the resulting error was found to vary with different invertase preparations. In some recent experiments an invertase preparation particularly subject to this error was used and as considerable accuracy in the experimental work was necessary for the purpose in view the results were of little value. An investigation was therefore undertaken of

[1] National Research Fellow.

[2] Nelson and Vosburgh, THIS JOURNAL, 39, 807 (1917).

the errors in the study of invertase action and a determination of how they may be reduced in magnitude.

Experimental.

The procedure used in the above mentioned investigation was the following. An invertase and a sugar solution were prepared separately in concentrations twice those at which the invertase and sugar were desired in the finished solution. Equal volumes of the two were mixed to start the reaction and samples taken from time to time. Enough hydrochloric acid to bring the hydrogen-ion concentration to the desired value was usually included in the invertase solution. The most probable points in the procedure at which loss of activity might occur are where the solution containing the invertase is diluted. Therefore an invertase solution was diluted in different ways and the activities of the resulting solutions were compared.

Dilution of Invertase with very Dilute Acid Solutions.—The concentration of acid was so chosen that if the dilute invertase were mixed with an equal volume of a solution containing 20 g. of sucrose per 100 cc., but no buffer, the hydrogen-ion concentration of the resulting solution would be about 4×10^{-5} *M*. The hydrogen-ion concentration of the dilute invertase was therefore a little less than 4×10^{-5} *M*. Five cc. of the stock invertase was added to about 80 cc. of a solution containing the amount of acid or other substance shown in Col. 2 of Table 1, and the solution made up to 100 cc. A sugar solution was prepared containing 5 g. of sucrose, 21.9 cc. of water, or water containing some buffer. Both solutions were brought to the temperature of the thermostat, 25° ± 0.01°, 25 cc. of the dilute invertase was added to the sugar solution and the reaction allowed to proceed for exactly 2 hours. A 25-cc. sample was then run into 5 cc. of 0.01 molar sodium carbonate solution, and the optical rotation determined. The solutions at the start of the reaction all contained 5 g. of sucrose, 1.25 cc. of the stock invertase and 5 cc. of citrate and hydrochloric acid buffer solution[3] in 50 cc.[4] The hydrogen-ion concentration was determined colorimetrically[5] in each experiment, and varied only to a negligible extent in the various experiments. The results are shown in Table I.

It is shown in Table I that dilution of definite amounts of invertase with small amounts of various substances present gives solutions which vary in activity according to the substance present during dilution. Thus the presence of hydrochloric or sulfuric acid causes a noticeable loss in

[3] Buffer No. 1. See below.

[4] The small amount of acid in the dilute invertase and the alkali which was sometimes included in the sugar solution to neutralize it were considered negligible.

[5] The procedure was that used by Nelson and Vosburgh, *loc. cit.* The values are based on the assumption that the hydrogen-ion concentration of 0.1 *M* hydrochloric acid is 0.0920 *M*.

TABLE I.

Dilution of Invertase in the Presence of Various Substances.

The invertase solution was prepared by diluting 5 cc. of the stock solution with about 80 cc. of a solution containing substances noted in Col. 2, and making up to 100 cc. with water. 25 cc. was added to the sugar solution to start the reaction.

The sugar solution contained 5 g. sucrose, a cc. of solutions noted in Col. 3 and $(21.9 - a)$ cc. of water.

A. Invertase No. 3.

Expt.	Invertase solution contains per 100 cc.	Sugar solution contains, a, cc. of buffer.	Conc. $H^+ \times 10^6$	Rotation after 2 hrs.	Change in 2 hrs.
9	[a] Water only	5	4.0	0.23	12.83
10		5	3.8	0.25	12.81
22	Water only	5	4.3	0.25	12.81
6			4.1	0.52	12.54
7	20 cc. buffer		3.8	0.32	12.74
8			3.8	0.32	12.74
26	10 cc. buffer	2.5	4.0	0.24	12.82
27		2.5	3.7	0.10	12.96
12	1.1 cc. 0.01 M HCl	5[b]	3.9	0.95	12.11
13		5[b]	3.7	1.11	11.95
B. Invertase No. 3A					
40	Water only	5	4.1	0.34	12.72
45	Water only	5	4.3	0.32	12.74
46	1.0 cc. 0.005 M H_2SO_4	5	4.3	1.20	11.86
47		5[b]	4.5	1.62	11.44
41	1.4 cc. 0.005 M Citric Acid	5[b]	4.3	0.79	12.27
42		5	4.3	0.81	12.25
43	2.3 cc. 0.01 M Acetic Acid	5	4.3	0.56	12.50
44		5	4.5	0.60	12.46
48	1.1 cc. 0.005 M Tartaric Acid	5	4.3	1.06	12.00
49		5	4.3	1.00	12.06
52	0.059 g. NaCl	5	4.3	0.61	12.45
53		5	4.3	0.72	12.35

[a] Brackets indicate that portions of the same dilute invertase solution were used in the experiments in the same bracket.

[b] Contains also 0.3 cc. of 0.01 N NaOH.

activity if the experiments in which the dilution is made with water only are taken as a standard. It is to be noted also that duplicate determinations in the case of dilution in the presence of hydrochloric or sulfuric acid agree poorly among themselves. Weak acids also cause losses in activity but duplicate determinations agree in this case. Different acids cause losses of different magnitudes.

The presence of a buffer causes a small loss in activity when all is included in the invertase solution and apparently a gain when half is in the invertase and half in the sugar. However, in both cases the results of duplicate

determinations do not agree well. It is strange that a citrate buffer in about 0.01 molar concentration should cause less loss in activity than citric acid at a much smaller concentration. Sodium chloride in 0.01 molar concentration acts much like citric acid.

Reproducibility of Dilution with Water.—The main conclusion to be drawn from Table I is that if an invertase solution is to be diluted with minimum loss in activity and in such a way that duplicate determinations of its activity will agree within less than 10 parts per thousand, the dilution must be made with pure water. The experiments listed in Table II show the degree of reproducibility of dilution with water. The procedure was like that of those experiments of Table I in which pure water was used for dilution of the invertase.

TABLE II.

Reproducibility of Dilution with Water.

Five-cc. portions of invertase No. 3 were diluted to 100 cc. with water and 25 cc. added to a sugar solution containing 5 g. of sucrose, 16.9 cc. of water and 5 cc. of buffer No. 1.

Expt.	22.	23.	24.	25.	9.	35.	38.
Conc. $H^+ \times 10^5$.....	4.3	3.9	...	4.0	4.0	4.0	4.0
Rotation after 2 Hrs.	0.25	0.25	0.23	0.22	0.23	0.28	0.23
Change in 2 Hrs.....12.81		12.81	12.83	12.84	12.83	12.78	12.83

It is evident from Table II that in the dilution of invertase solutions with water duplication of results within 5 parts per thousand is possible. However dilution of 5 cc. of the stock solution to 100 cc. only is considered. Nelson and Hitchcock[6] have found that poor duplication resulted after a dilution considerably greater than this.

Loss in Activity on Adding Invertase to a Sugar Solution.—The total loss in activity before the beginning of the reaction must be the sum of the loss due to the preliminary dilution of the invertase and that taking place on mixing with the sugar solution. In drawing the conclusions above it has been assumed that the second loss is either constant or small compared with the first. It seems reasonable to conclude from Table II that if the presence of a citrate buffer has any effect at this point the effect is reproducible within at most 5 parts per thousand and therefore that the above assumption is justified. The effect of some other sources of hydrogen ion when used instead of the buffer is shown in Table III. The experiments were carried out according to the same procedure as those of Table I, the invertase being diluted with water only and the substances noted in Col. 2 being present in the respective sugar solutions.

In the experiments of Table III the differences in the change in rotation can be ascribed to changes in the activity of the invertase at the time of mixing with the sugar solution or to different rates of reaction. The

[6] Private communication

TABLE III.

Effect of Various Sources of Hydrogen Ion when Included in the Sugar Solution Previous to Adding the Invertase.

Invertase solution : 5 cc. of Invertase 3 A per 100 cc. diluted with water only.
Sugar solution : 5 g. of sucrose, (21.9—*a*) cc. of water and *a* cc. of solution in Col. 3.

Expt.	Sugar solution contains, Cc.	Solution.	Conc. $H^+ \times 10^5$.	Rotation after 2 hrs.	Change in 2 hrs.
45	5	Buffer No. 1	4.3	0.32	12.74
70	5	" " 1	4.0	0.31	12.76
64	0.25	0.01 *M* HCl	2.8	0.37	12.70
65	0.25	0.01 *M* HCl	3.8	0.42	12.65
66	0.25	0.005 *M* H_2SO_4	3.6	0.40	12.67
67	0.25	0.005 *M* H_2SO_4	4.0	0.39	12.68
68	0.35	0.005 *M* Citric Acid	3.5	0.45	12.62
69	0.35	0.005 *M* Citric Acid	3.7	0.49	12.58
73	0.6	0.01 *M* Acetic Acid	3.5	0.59	12.48
74	0.6	0.01 *M* Acetic Acid	3.5	0.59	12.48
75	0.3	0.005 *M* Tartaric Acid	3.7	0.74	12.33
76	0.3	0.005 *M* Tartaric Acid	3.5	0.70	12.37

latter explanation seems less probable. Retardation similar to that found by Fales and Nelson[7] to be caused by sodium chloride cannot have taken place since the concentrations of electrolyte were small, the hydrogen-ion concentration was the optimum, and the largest concentration of electrolyte was present in the experiment in which the fastest reaction took place. Since dilution of the stock invertase with very weak electrolyte solutions is attended by a loss in activity it is reasonable to expect that dilution of an invertase solution with a sugar solution containing the same electrolyte would also be attended by a loss in activity. Therefore it is assumed that the differences in reaction velocity in the various experiments were chiefly due to changes in activity of the invertase on mixing with the sugar solution.

The results of dilution with a sugar solution containing various sources of hydrogen ion are similar to the results of dilution of the stock invertase with water containing the same substances. A greater loss in activity takes place when free acids are the source of hydrogen ion than when a buffer is used. However the loss in activity caused by the strong acids is here less than that caused by the weak ones. The magnitude of the losses is in most cases much smaller than those in the experiments of Table I. This might be explained as due to the smaller amount of dilution, 25 cc. to 50 cc. as compared with 5 to 100, or it might be explained as the result of "protective action"[8] of the sugar.

[7] Fales and Nelson, THIS JOURNAL, **37**, 2769 (1915).

[8] O'Sullivan and Thompson, *J. Chem. Soc.*, **57**, 900 (1890), have shown that sucrose protects invertase from destruction by hot water; and Hudson, THIS JOURNAL, **32**, 988, 1354 (1910) has shown that fructose also protects it from destruction by hot water as well as by acids and alkalies, and that sucrose protects it from destruction by alcohol.

That sucrose has such a protective action in this case is shown in the experiments of Table IV. In these, 5 cc. of the stock invertase was added directly to 195 cc. of a solution containing 20 g. of sucrose, and either 20 cc. of buffer or the quantity of acid necessary to make the hydrogen-ion concentration of the completed solution about $4 \times 10^{-5} M$. The concentrations of invertase, sucrose, and hydrogen ions are consequently the same as in the experiments of Table III, only the total volume and manner of mixing differing.

TABLE IV.

Rate of Hydrolysis when No Preliminary Dilution is Made.

The sugar solution contained 20 g. of sucrose, $(182.6 - a)$ cc. of water and a cc. of acid or buffer as noted in Col. 3. 5 cc. of Invertase 3 A was added.

Expt.	Sugar solution contains, Cc.	Solution.	Conc. H^+ $\times 10^5$.	Rotation after 2 hrs.	Change in 2 hrs.
60	20	Buffer No. 1	4.0	0.17	12.89
61	20	Buffer No. 1	4.1	0.17	12.89
78	20	Acetate Buffer	4.3	0.22	12.84
83	20	Acetate Buffer	4.1	0.29	12.77
87	1.4	0.005 M H_2SO_4	4.7	0.45	12.61
80	2.3	0.005 M Citric Acid	4.4	0.42	12.64
86	2.1	0.005 M Citric Acid	5.1	0.39	12.67
81	4.8	0.01 M Acetic Acid	5.5	0.39	12.67
84	4.3	0.01 M Acetic Acid	5.1	0.45	12.61

The results given in Table IV are similar to those in Table III. Dilution with a sugar solution containing a very weak electrolyte evidently has less destructive action than dilution with a very weak electrolyte in absence of sugar. Expts. 60 and 61 as compared with Expts. 45 and 70 in Table III show that in the latter there is a loss in activity on dilution of the invertase with water.

It seems reasonable that the best procedure to adopt in the study of invertase action is that which will give the fastest reaction, other conditions being the same. The experiments of Tables III and IV point out therefore that the invertase should be used without preliminary dilution if possible, and that buffers are preferable to acids as the source of hydrogen ions.

Experiments with Other Invertase Preparations.—Experiments similar to some of the above experiments were carried out with two other invertase preparations, Nos. 2 and 8, and the results are given in Tables V, VI, and VII. The procedure was essentially the same as in the experiments of Tables I, III and IV respectively.

It is shown in Table V that dilution in presence of hydrochloric acid has some destructive action on Invertases 8 and 2 but the amount is noticably less than that in the case of Invertase 3. In case of Invertase 2 it is probable that the amount of acid used was not equal with respect to the dilution effect to that used with Invertases 3 and 8, since more

TABLE V.

Dilution of Invertase Preparations 2 and 8 with Very Weak Acid Solutions.

Procedure as in experiments of Table I. Sugar solutions contained 5 g. of sucrose, 16.9 cc. of water and 5 cc. of buffer.

A. Invertase No. 8.

Expt.	Invertase solution contains per 100 cc. Cc.	Solution.	Buffer No. [b].	Conc. $H^+ \times 10^5$.	Rotation after 2 hrs.	Change in 2 hrs.
90			1	3.8	—1.09	14.14
96			2	4.4	—1.07	14.12
98			2	4.5	—1.07	14.12
122			3	4.5	—1.10	14.15
91	0.8	0.01 *M* HCl	1	4.5	—1.00	14.05
92			1	4.3	—1.01	14.06
101	1.1	0.01 *M* HCl [a]	2	4.0	—0.74	13.79
124	1.1	0.01 *M* HCl [a]	3	4.3	—0.82	13.87

B. Invertase No. 2. 10 cc. of stock solution per 100 cc. of dilute invertase.

125			3	4.3	4.79	8.33
132			3	4.7	4.84	8.28
138			3	4.5	4.85	8.27
127	1.1	0.01 *M* HCl [a]	3	4.3	4.85	8.27
135	1.1	0.01 *M* HCl [a]	3	4.3	4.87	8.25

[a] Sugar solution contained 0.3 cc. of 0.01 *M* NaOH, 16.6 cc. of water and 5 cc. of buffer to 5 g. of sucrose.

[b] Citrate and hydrochloric acid buffers.

acid was required to bring a solution containing Invertase 2 in the concentration used to a hydrogen-ion concentration of 4×10^{-5} *M* than in the case of Invertases 3 and 8. The magnitude of the dilution effect on Invertase 8 seems to be somewhat dependent on the hydrogen-ion concentration, the loss in activity being larger when the acid concentration is larger. Therefore although the observed loss in activity by Invertase 2 is small, it probably would be somewhat larger if an amount of acid equivalent to that in the experiments with Invertase 8 were present.

It can be concluded from Table V that dilution of any invertase in presence of hydrochloric acid is likely to result in loss in activity, but that the magnitude of the losses under the same conditions differs in different invertase preparations. Invertase solutions may be classified according to stability, the more stable ones being those like Nos. 2 and 8 which lose the least on dilution and the less stable being those like No. 3. Dilution with water is reproducible within 2 parts per thousand in the case of Invertases 8 and 2.[9]

[9] The rather large difference between Expts. 125 and 132 with Invertase 2 may have been due to decrease in activity of the stock solution during the 11 days which elapsed between the two experiments, the solution having been removed from the ice-box during part of 1 day. In view of this the experiments with Invertase 2 numbered above 128 should be compared with No. 128 rather than with No. 125, and those numbered below 125 should be compared with the latter.

TABLE VI.

Effect on Invertases 2 and 8 of Acids when Present in the Sugar Solution Previous to Adding the Invertase.

Procedure as in the experiments of Table III except that a 10-cc. portion of Invertase 2 was used.

A. Invertase No. 8.

Expt.	Sugar solution contains, Cc.	Solution.	Conc. $H^+ \times 10^6$.	Rotation after 2 hrs.	Change in 2 hrs.
90	5	Buffer No. 1	3.8	—1.09	14.14
96	5	Buffer No. 2	4.4	—1.07	14.12
93	0.25	0.01 *M* HCl	3.4	—1.03	14.08
97	0.27	0.01 *M* HCl	3.6	—1.07	14.12
123	0.30	0.01 *M* HCl	5.0	—1.00	14.05
99	0.38	0.005 *M* Citric Acid	3.7	—1.05	14.10
100	0.38	0.005 *M* Citric Acid	3.7	—1.05	14.10

B. Invertase No. 2.

125 [a]	5	Buffer No. 3	4.3	4.79	8.33
132	5	Buffer No. 3	3.7	4.84	8.28
138	5	Buffer No. 3	4.5	4.85	8.27
126	0.35	0.01 *M* HCl	3.7	4.91	8.21
139	0.37	0.01 *M* HCl	4.2	4.94	8.18
140	0.52	0.005 *M* Citric Acid	4.3	4.86	8.26
141	0.52	0.005 *M* Citric Acid	4.4	4.87	8.25

[a] See note above.

Table VI shows that the conclusions drawn from Table III can be extended to apply to other invertase preparations, except as to the magnitude of the losses in activity under the various conditions. The change in rotation in the experiments with citric acid as the source of hydrogen ions is only 2 to 3 parts per thousand less than in the experiments in which buffer is used. With the more stable invertase preparations therefore citric acid can be used as the source of hydrogen ions instead of a buffer. Hydrochloric acid is less reliable as to reproducibility and should not be used when results of high precision are desired.

TABLE VII.

Rate of Hydrolysis when No Preliminary Dilution is Made.

The procedure was like that of the experiments of Table IV. Five cc. of invertase was used in each experiment.

Expt.	Invertase. No.	Buffer. No.	Cc.	Sucrose. G.	Water. Cc.	Conc. $H^+ \times 10^6$.	Rotation after 2 hrs.	Change in 2 hrs.
105	8	2	20	20	162.6	4.3	—1.11	14.16
106	8	2	20	20	162.6	4.6	—1.11	14.16
136	2	3	10	10	78.8	4.3	4.82	8.30
137	2	3	10	10	78.8	4.3	4.83	8.29

Comparison of the experiments in Table VII in which no preliminary dilution was made with those of Table V in which preliminary dilution was

made shows that in the case of Invertases 8 and 2 only small losses in activity took place on dilution with water, the change in rotation being about 3 parts per thousand[10] larger than in the case in which no preliminary dilution was made. The difference between stable and unstable invertase preparations is noticeable in the amount of loss in activity caused by dilution with distilled water.

Loss in Activity by Dilute Invertase on Standing.—In the above experiments the dilute invertase solutions were used within 2 hours after preparation. It was found that such solutions when kept at 25° lost activity as time went on. For example in an experiment with Invertase 3A the change in rotation using the freshly prepared dilute invertase was 12.78°, while that after the solution had been kept for one day was 12.69°. The activity decreased regularly and after 11 days the change in rotation was 12.38°. When a similar solution was kept in the ice-box the loss in activity was much slower, but still appreciable. In this case the change in rotation when the invertase was freshly diluted was 12.48° and after 20 days was 12.35°.

A similar loss in activity takes place in dilute solutions of other invertase preparations. In the case of the dilute Invertase 8 used in Expt. 96[11] determinations after 1 and 4 days' standing at 25° gave respectively 13.98° and 13.82° change in rotation as compared with 14.12° in Expt. 96. The dilute Invertase 8 used in Expt. 122[11] was kept in the ice-box for 12 days after which the change in rotation was 13.99° as compared with 14.15° in Expt. 122. In the case of Invertase 2 the dilute solution of Expt. 138, giving in that experiment a change in rotation of 8.27° gave after 5 days in the ice-box 8.03°.

Hudson and Paine[12] claim that destruction of invertase by acids or alkalies is only appreciable when the acid or alkali concentration is 0.01 molar or greater. The hydrogen-ion concentration of a dilute solution of Invertase 3A was found to be 5.77 when freshly diluted, and after 5 days 5.84. This would indicate that the loss in activity is due to some other cause than the destructive action of acids or alkalies. On the other hand when a small amount of acid was present in the dilute invertase it was found that the loss in activity in two days was much greater than the loss during the same time by a solution made up without acid.

Loss in Activity by Stock Solutions.—Fales and Nelson[7] reported that the stock solution used by them did not lose in activity to an appreciable extent in a year. However, the above results for the dilute invertase suggest that the stock solutions may have lost in activity also. In Table

[10] Expts. 105 and 106 are compared with 96 and 98 since the same buffer was used in all.

[11] Table V.

[12] Hudson and Paine, THIS JOURNAL, **32**, 774 (1910).

VIII are collected the results of several experiments on the activity of Invertases 3A and 8 over a period of more than 3 months. The procedure in these experiments was like that of the experiments of Table II. It is shown that these stock solutions lose slowly in activity. The more stable invertase preparations change more slowly than the less stable ones. The decrease in activity in Invertase 3A is a little more than half the rate at which the dilute solution which was kept in the ice-box changed. In the case of Invertase 8 the stock solution changed much more slowly than the dilute solution.

TABLE VIII.

Activity of Stock Invertase Solutions.

Procedure as given in Table II.

Expt.	Invertase.	Date.	Buffer. No.	Conc. $H^+ \times 10^5$.	Rotation. after 2 hrs.	Change. in 2 hrs.
40	3 A	Aug 2	1	4.1	0.34	12.72
54	3 A	" 5	1	3.9	0.29	12.77
70	3 A	" 10	1	4.0	0.31	12.76
118	3 A	" 26	3	4.3	0.41	12.66
149	3 A	Oct. 13	3	4.1	0.63	12.44
151	3 A	Nov. 18	3	4.5	0.76	12.31
90	8	Aug. 18	1	3.8	—1.09	14.14
122	8	" 27	3	4.5	—1.10	14.15
150	8	Oct. 13	3	4.1	—1.02	14.07
152	8	Nov. 18	3	4.3	—0.98	14.03

In using an invertase solution care should be taken to make a complete series of experiments in as short a time as possible and to measure the activity of the invertase at least at the beginning and end of the series if the series is long. Furthermore, considering also the previous action, it follows that if it is necessary to dilute a stock solution, it should be freshly diluted just previous to using.

In making up Tables I to VII only the results of experiments with any one invertase which were made within such time limits that the deterioration meanwhile was negligible were included in any one section of a table.

Experimental Details.

Measurement of the Initial Rotation.—In using invertase inactivated by boiling to make blank determinations of the initial rotation the assumption is made that boiled invertase has practically the same specific rotation as the active invertase. Therefore the optical rotation was determined of boiled and unboiled portions of the invertase preparations used in this investigation. Five-cc. portions of Invertases 3A and 8 and a 10-cc. portion of Invertase 2 were made up to 100 cc. with water. Twenty-five-cc. samples were taken and run into 25 cc. of water and 10 cc. of 0.01 *M* sodium carbonate solution, this procedure being similar to that of the actual experiments except that the sugar was omitted. A portion of the 100 cc. of dilute invertase solution was brought to the boiling point and allowed to cool and the sampling repeated. The optical rotations of the solutions so prepared were determined, and found to be 0.05, 0.02 and 0.12 for the active Invertases 3A, 8 and 2 respectively, and 0.05, 0.03, 0.12 for the inactive

invertases, respectively. Invertase inactivated by boiling evidently has for practical purposes the same specific rotation as active invertase. It is also shown that a separate blank determination must be made for each invertase preparation.

Blank determinations to apply to the above experiments were made as follows. Sugar solutions were prepared containing 5 g. of sucrose and 21.9 cc. of water, or water and buffer as noted below. Five-cc. portions of Invertases 3A and 8 or 10-cc. portions of Invertase 2 were diluted with water and heated to boiling. The invertase solutions were allowed to cool, made up to 100 cc. and 25-cc. portions were added to the sugar solutions as in the actual experiments. Twenty-five-cc. samples were then run into 5-cc. portions of 0.1 *M* sodium carbonate solution and the optical rotations determined. In the experiments using Invertase 3A two different sucrose preparations were used, the first in Expts. 6 to 52 and the second in the experiments numbered higher than 52. Blank determinations of the initial rotation were made for Invertase 3A with both. The second sucrose preparation was used in the experiments with the other invertase preparations. The initial rotations were as follows:

A. Invertase 3A.

Sucrose No.....	1	1	1	1	2	2
Buffer, cc.....	5	5	..	...	5	5
0.005 *M* H_2SO_4, cc.	...	...	0.3	0.3	...	...
Rotation.....	13.06	13.06	13.06	13.07	13.07	13.07

B. Invertase 8.

Rotation.....	13.05	13.05

C. Invertase 2.

Rotation.....	13.12	13.13

The initial rotation for the experiments like those of Table IV was determined for Invertase 3A using the same procedure as in actual experiments but with inactive invertase. It was found to be 13.06° which is 0.01° lower than with the above procedure. Since 0.01° is about the limit of accuracy in reading the polariscope the initial rotations in the case of the experiments of Table VII were taken as the same as those given in B and C above.

Determination of the Change in Rotation.—The invertase solution was added to the sugar solution by means of a calibrated pipet and the time of the start of the reaction considered as the mean time of delivery. The reaction was allowed to proceed for 2 hours and then a 25-cc. sample was withdrawn and run into 5-cc. of 0.01 *M* sodium carbonate solution[13] at a time such that the mean time of delivery would be exactly 2 hours after the start of the reaction. The rotation of the sample was determined after at least 10 minutes and before it had stood 2 hours, by means of a Schmidt and Haensch polariscope which could be set and read to about 0.01°. Light from a mercury-vapor lamp filtered by a Wratten No. 74 filter was used for the polariscopic measurements. The temperature of the sample was maintained at 25° during the determination of the rotation by means of the thermostat described by Nelson and Beegle.[14] The zero point of the polariscope was determined at least once on each day on which determinations were made, using one of the polariscope tubes filled with distilled water. The cover glasses were tested occasionally by turning the tubes while making readings but no optical rotation was ever observed in the 4 cover glasses used throughout this investigation.

Preparation of the Invertase.—Invertase 3 and Invertase 8 were prepared in this laboratory by Mr. D. I. Hitchcock and Invertase 2 by Dr. H. L. Simons, the author being indebted to both for the invertase used in this investigation. Nos. 2 and 3 were prepared from autolysed yeast which had been standing for several years. No. 8 was

[13] Hudson, THIS JOURNAL, **30**, 1564 (1908).

[14] Nelson and Beegle, *ibid.*, **41**, 559 (1919).

prepared from fresh yeast. Autolysis, precipitation of the proteins and dialysis were carried out as described by Nelson and Born.[15] The invertase was however not precipitated from the dialyzed solutions, the latter being used as the "stock solutions." Invertase 3A was a small portion of Invertase 3 which was removed and kept in a smaller bottle for convenience. The apparent difference in activity between the two may have been due to deterioration during the time that elapsed between the experiments with Invertase 3 and those with 3A. All stock solutions were kept in an ice-box.

Preparation of the Sucrose.—Rock candy known to have been made from the best commercial sucrose was dissolved and reprecipitated by addition of alcohol as described by Cohen and Commelin.[16] It was dried over conc. sulfuric acid at about 50° and preserved in glass-stoppered bottles sealed with paraffin.

Preparation of the Buffers.—Buffers 1 and 3 were prepared by adding 197 cc. of 0.1 *M* hydrochloric acid to 303 cc. of secondary sodium citrate freshly prepared as described by Sorensen.[17] Buffer No. 2 was prepared similarly using a citrate solution prepared from *N* sodium hydroxide solution which had stood for some time and had dissolved some silica from the bottle. The acetate buffer was prepared by adding 655 cc. of 0.1 *M* acetic acid to 345 cc. of 0.1 *N* sodium acetate.

Experiments similar to those of Table II with Invertase 3A using the different buffer solutions are shown in Table IX. The slight amount of impurity in buffer No. 2 was found to have an appreciable effect on the velocity of hydrolysis. Expts. 117 and 118 show that the velocity when buffer No. 2 is used is less than when No. 3 is used. The apparent difference between the effects of Buffers 1 and 3 on the velocity of hydrolysis can be explained by deterioration of the invertase during the time between Expts. 70 and 118.

The acetate buffer gives a velocity of hydrolysis which is a little less than that when a citrate buffer is used as Expts. 70, 71 and 72 in Table IX and 60, 61, 78 and 83 in Table IV show.

TABLE IX.

Effect of Different Buffers on the Activity of Invertase.

Procedure as in the experiments of Table II.

Expt.	Buffer.	Date. Aug.	Conc. $H^+ \times 10^5$.	Rotation after 2 hrs.	Change in 2 hrs.
40	Citrate No. 1	2	4.1	0.34	12.72
70	1	10	4.0	0.31	12.76
95	2	19	3.8	0.52	12.55
107	2	23	4.3	0.49	12.58
117	2	26	4.3	0.59	12.48
118	3	26	4.3	0.41	12.66
71	Acetate	10	4.3	0.40	12.67
72		10	4.5	0.36	12.71

The differences in the effect of the different citrate buffers on Invertase 8 are shown in Table V to be very small. Here again the difference between a stable and an unstable invertase preparation is noticeable, the former being preferable.

Summary.

1. Invertase solutions are subject to loss in activity when diluted. The loss varies with the substances present in the water with which the invertase is diluted.

[15] Nelson and Born, THIS JOURNAL, **36**, 393 (1914).

[16] Cohen and Commelin, *Z. physik. Chem.*, **64**, 1 (1908).

[17] Sorensen, *Biochem. Z.*, **21**, 131 (1909).

2. Dilution with distilled water is attended by less loss than is the case in dilution with very weak acids, and is reproducible for practical purposes if the dilution is not too great.

3. When an invertase solution is added to a solution containing sucrose, losses in activity due to dilution are less than when the invertase is diluted by a similar solution not containing sucrose.

4. The velocity of hydrolysis of sucrose by invertase is greater when a citrate buffer or acetate buffer is used as the source of hydrogen ions than when citric or acetic acid respectively is so used.

5. With different invertase preparations the magnitude of the losses in activity on dilution varies. Invertase solutions may be designated as more or less stable according to the magnitude of such losses in activity.

6. Both dilute and stock solutions of invertase lose strength on standing, the former faster than the latter. Stock solutions of the more stable invertase preparations lose activity more slowly than stock solutions of the less stable invertase preparations.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY.]

ADDITION REACTIONS OF THE CARBONYL GROUP INVOLVING THE INCREASE IN VALENCE OF A SINGLE ATOM.

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Received April 29, 1921.

The addition reactions of the phosphorus halides, which have been described in previous papers,[1] are of a different type from the usual addition reactions of simple or conjugated carbonyl compounds. They involve the formation of a cyclic compound by reason of the increase in valence of a single atom, while in most addition reactions of carbonyl compounds the reagent seems to have dissociated into two parts which subsequently become attached to the carbon and oxygen atoms. The Grignard reagent, hydrocyanic acid, and sodium bisulfite may be mentioned as common examples of this usual type of reaction.

$$R_2C{=}O + AB \rightarrow R_2C(A){-}OB$$

These common reagents react with α,β unsaturated ketones and aldehydes in much the same manner. When they combine with the conjugated system, the two parts of the addendum attach themselves in the 1,4 position to the oxygen and carbon atoms. The molecule of the reagent apparently undergoes cleavage into the same fragments in the case of both 1,2 and 1,4 additions.

[1] THIS JOURNAL, **39**, 2679 (1917); **42**, 830, 2337 (1920); **43**, 1665,1667 (1921).