

of both the possible diphenyl-bromophthalides was obtained from 4-bromophthalic anhydride. Oddy, working at McMaster University, has obtained good yields of the diphenyl-tetrahalogen-phthalides from the tetrahalogen-phthalic anhydrides.

Summary.

1. Each of the two bromophthalic anhydrides has been found to react with benzene and aluminum chloride forming benzoyl-bromobenzoic acids. The 3-anhydride yielded only one acid while the 4-anhydride gave two in approximately equal quantities.

2. Under the conditions favorable to the production of diphenyl-bromophthalides, the 3-anhydride gave no diphenyl-bromophthalide while the 4-anhydride gave two.

3. A new method is described for the preparation of 3-bromophthalic acid and detailed directions given for the preparation of 1,6-dibromo-2-naphthol and 4-bromophthalic acid.

4. Seven new compounds have been prepared: 2-benzoyl-4-bromobenzoic acid, m. p. 193°; diphenyl-3-bromophthalide, m. p. 131°; diphenyl-4-bromophthalide, m. p. 115-6°; diphenyl-5-bromophthalide, m. p. 186°; mixed anhydride of 6-benzoyl-2-bromobenzoic acid and acetic acid, m. p. 168.5°; mixed anhydride of 2-benzoyl-4-bromobenzoic acid and acetic acid, m. p. 83-7°; and mixed anhydride of 6-benzoyl-3-bromobenzoic acid and acetic acid.

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THE ACTIVITY OF ADSORBED INVERTASE.

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The action on sucrose solutions of invertase adsorbed on charcoal and on gelatinous aluminum hydroxide was studied by Nelson and Griffin¹. They reported that in their experiments a given quantity of enzyme exhibited the same activity when adsorbed on a solid in the bottom of the reaction vessel as when uniformly distributed throughout the solution. The importance of this observation and its bearing on the true nature of

¹ Nelson and Griffin, *THIS JOURNAL*, **38**, 722, 1109(1916).

enzyme action have been pointed out by Bayliss², Lewis³, Falk⁴ and others, and have led to the present further study of this phenomenon.

In some preliminary experiments with different charcoals it was found that in all cases where the invertase was completely adsorbed (or nearly so) there was a marked retardation of the hydrolysis. One charcoal was tried which caused very little retardation, but it was found that in this case the invertase was adsorbed only to a small extent. In order to determine whether the invertase was largely adsorbed or not, a mixture of sugar solution, adsorbent and invertase was prepared, shaken, and filtered about 10 or 15 minutes after the addition of the invertase. The absence of further change in the optical rotation of the filtrate indicated that the invertase had been completely adsorbed; much change in the rotation of the filtrate indicated incomplete adsorption. The retardation observed in the presence of the adsorbent could not have been due to changes in the hydrogen-ion concentration, since this was kept constant in all the experiments, both with and without adsorbent, by means of buffers. The result of a typical experiment with a charcoal of good adsorbing power are given in Table I.

TABLE I.
Change in rotation.

Time, hours.	Filtrate.	Control (no charcoal).	Solution with charcoal.
18	0.40°	7.14°	2.03°
25.3	0.64	8.21	2.59
42.3	0.91	8.94	3.68

On testing the activity of invertase adsorbed on aluminum hydroxide, it was found that here too there was considerable retardation. It was thought that this might be due to the mechanical removal of the enzyme from the solution by the settling of the adsorbent. To test this explanation a mechanical stirrer was employed, which prevented the aluminum hydroxide from settling. It was found that the stirring prevented the greater part of the retarding action, as is shown by the results in Table II.

TABLE II.

Expt.	Sucrose conc., g. per 100 cc.	Time, Hours.	Filtrate.	Change in rotation.		
				Control.	With Al(OH) ₃ .	
				Stirred.	Not stirred.	
20	5	1	0.00°	4.23°	3.82°	2.25°
21	10	1	0.01	4.53	4.18	3.61

From these experiments it was concluded that when an adsorbent is used without stirring, the rate at which the sucrose reaches the invertase is governed by the rate of diffusion of the sucrose down to the bottom of the vessel. Accordingly it was thought that if the action of the invertase

² Bayliss, "The Nature of Enzyme Action," 4th Ed., 1919, p. 112.

³ Lewis, "A System of Physical Chemistry," 2nd Ed., Vol. I, 1918, p. 460.

⁴ Falk, "The Chemistry of Enzyme Action," 1921, p. 61.

could be made even slower than the diffusion of the sucrose to the bottom of the reaction bottle, then there should be no retardation by the aluminum hydroxide. This was tried with 3 different invertase preparations by diluting the invertase to different degrees, with the results shown in Table III.

TABLE III.

Effect of Invertase Concentration on Retardation by Aluminum Hydroxide.

Expt.	Invertase conc. cc. per 100 cc. Prep. No. la	Time. Hours.	Filtrate.	Change in rotation.		Retardation by Al(OH) ₃ , % of change in control.
				Control.	With Al(OH) ₃ .	
22	2.50	18	0.00°	3.10°	2.78°	10
25	1.25	18	0.07	1.51	1.52	00
26	0.50	18	0.00	0.59	0.60	
Prep. No. 8.						
27	1.00	1	0.05	6.79	4.68	31
28	0.10	12	0.05	6.97	5.58	20
31	0.05	12	0.00	3.66	3.20	13
32	0.025	24	0.01	3.65	3.47	5
30	0.01	24	0.01	1.43	1.45	0
Prep. No. 10.						
41	2.50	24	0.11	3.83	3.38	12
43	1.25	24	0.05	1.38	1.29	7
44	0.50	24	0.02	0.75	0.73	3
44	0.50	48	0.01	1.38	1.37	1
44	0.50	96	0.01	2.45	2.47	0

It seems from these figures that the absence of retardation observed by Nelson and Griffin is not general, but represents only a very special case. Probably in most of their work the action of the enzyme was slower than the rate of diffusion of the sucrose. The slow rate of reaction in their second series of experiments⁵ indicates that this explanation may apply there.

In some of the experiments with charcoal reported by Griffin and Nelson in their first paper,⁶ the rate of reaction seems not to have been very slow. Since they gave no measurements of the inverting power of the filtrates in these experiments, it is possible that the absence of retardation may have been due to incomplete adsorption of the invertase by the charcoal. The present authors are unable to explain the earlier experiment⁷ with aluminum hydroxide at hydrogen ion concentration 5.0×10^{-7} in which it was shown that the invertase was completely adsorbed. Repetitions of this experiment at about the same rate of inversion have indicated a retardation by aluminum hydroxide.

⁵ *Loc. cit.*, p. 1110.

⁶ *Ibid.*, p. 727.

⁷ *Ibid.*, p. 729.

Experimental Details.

Materials.—The invertase was prepared from yeast by the method of Nelson and Born⁸ omitting the second and final precipitations by alcohol. Preparation 10 was obtained by dissolving 0.570 g. of the solid Invertase A of Nelson and Vosburgh⁹ in 500 cc. of distilled water.

Suspensions of aluminum hydroxide prepared from the chloride could not be used; the precipitate so made either clogged the filter or ran through in a turbid colloidal suspension and did not completely adsorb the invertase. The aluminum hydroxide finally used was prepared from alum as described by Welker and Marshall,¹⁰ and was washed repeatedly by decantation and also by dialysis for 2 days against running water. The suspension contained 0.0205 g. of aluminum oxide in 5 cc. In each experiment 100 cc. of the reaction mixture contained 5 cc. of this suspension.

The charcoal used in the experiment of Table I was an imported decolorizing powder; 100 cc. of the reaction mixture contained 0.30 g. of charcoal. In other experiments samples of this charcoal which had been boiled with hydrochloric acid or ammonium hydroxide and then washed caused the same retardation as the untreated charcoal.

The cane sugar was used the best commercial grade, as was that used by Nelson and Griffin.

Control of the Hydrogen-ion Concentration.—The buffer used in the experiments with charcoal was a mixture of secondary sodium citrate and hydrochloric acid.¹¹ It was found that the aluminum hydroxide did not completely adsorb the invertase in the presence of a citrate buffer at $P_H = 4.45$ (or a phosphate buffer at $P_H = 6.3$); accordingly for the experiments with aluminum hydroxide a buffer mixture of acetic acid and sodium acetate was prepared.¹² In all experiments the reaction mixture was 0.01 *M* with respect to total electrolyte. In the experiments of Tables I, II, III the hydrogen-ion concentration was $10^{-4.4}$ to $10^{-4.6}$ moles per liter, which is the optimum for invertase action.¹³ The hydrogen-ion concentration was measured by the colorimetric method using α -naphthyl-amino-azo-*p*-benzene sulfonic acid as indicator with citrate standards, which were standardized electrometrically using the saturated potassium chloride calomel cell.¹⁴ The hydrogen-ion concentrations were based on 0.1000 *M* hydrochloric acid as a standard, its ionization¹⁵ being taken as 91.7% at 37°.

Procedure.—The method of experimentation was similar to that of Nelson and Vosburgh,⁹ but in accordance with the recommendation of Vosburgh¹⁶ the dilution of the invertase with anything except water was avoided until the time for starting the reaction. The invertase was diluted with distilled water to twice the strength required, and to start the reaction, it was mixed with an equal volume of a double strength

⁸ Nelson and Born, *THIS JOURNAL*, **36**, 393 (1914).

⁹ Nelson and Vosburgh *ibid.*, **39**, 790 (1917).

¹⁰ Welker and Marshall, *ibid.*, **35**, 822 (1909).

¹¹ Sorensen, *Compt. rend. Lab. Carlsberg*, **8** (1909); *Biochem. Z.*, **21**, 131 (1909).

¹² Michaelis, "Die Wasserstoffionenkonzentration," Berlin, 1914, p. 180.

¹³ Fales and Nelson, *THIS JOURNAL*, **37**, 2770 (1915), and others.

¹⁴ Fales and Mudge, *ibid.*, **42**, 2434 (1920).

¹⁵ A. A. Noyes, "The Electrical Conductivity of Aqueous Solutions," 1907, p. 339.

¹⁶ Vosburgh, "Some Errors in the Study of Invertase Action," *THIS JOURNAL*, **43**, 1693 (1921).

sugar solution containing buffer, either with or without charcoal or aluminum hydroxide. The concentration of sucrose in the reaction mixture was 10 g. per 100 cc., except in Table I and Expt. 20, Table II, where it was 5 g. per 100 cc. The inversions were allowed to take place in Nonsol glass bottles in a thermostat at $37^{\circ} \pm 0.02^{\circ}$. Bacterial action was avoided by keeping the solutions saturated with toluene. The reaction was stopped and mutarotation hastened by the use of sodium carbonate,¹⁷ a 25cc. sample being added to 5 cc. of 0.1 M sodium carbonate solution. For the measurements of rotation the solutions were put into 200mm. tubes in the thermostat described by Nelson and Beegle,¹⁸ which remained at $25^{\circ} \pm 0.05^{\circ}$. Readings were taken with a Schmidt and Haensch polarimeter which could be read and set to 0.01°. Light of wave length 546 $\mu\mu$ was used, being obtained from a mercury-vapor arc lamp and purified by a Wratten filter, No. 74.

Initial rotations were measured in samples in which the sodium carbonate was added before the invertase, which was thus rendered inactive so that no inversion took place. Identical values for the initial rotation were obtained in samples in which the aluminum hydroxide was mixed with the sugar solution before measuring out a sample of the latter and in others in which no aluminum hydroxide was used; this indicates that the volume occupied by the precipitate was negligible, and that no appreciable amount of sugar was adsorbed by it.

Solutions for the control experiments were made up with aluminum hydroxide, which was removed by filtration before the addition of the invertase. To determine whether the invertase was really adsorbed under the conditions of each experiment, a duplicate solution was prepared from which the adsorbent was filtered off about 10 or 15 minutes after the beginning of the reaction. Then a sample was taken, and the completeness of adsorption was determined by the absence of further change in rotation from that time on for a time equal to the duration of the main experiment.

In working with fairly rapid inversions, such as those in Table II, it was possible to obtain duplicate results which checked to 0.02° or better. With very dilute invertase, however, the agreement of the duplicates was worse, some differing by as much as 0.1°. This irreproducibility seems to be due to the effect of dilution noticed by Vosburgh.¹⁶

Summary.

1. A comparison has been made of the amount of sucrose hydrolyzed in a given time by invertase combined with an adsorbent and not combined but uniformly distributed throughout the solution.

¹⁷ Hudson, *THIS JOURNAL*, **30**, 1564 (1908).

¹⁸ Nelson and Beegle, *ibid.*, **41**, 559 (1919).

2. When all conditions were the same except the presence or absence of the adsorbent and the velocity of hydrolysis was relatively large, then the amount hydrolyzed in a given time was less in the presence of the adsorbent.

3. The decrease in rate appears to be due largely to the uneven distribution of the invertase in the reaction mixture, because by preventing the settling of the adsorbent combined with the enzyme the extent of the retardation was diminished.

4. When the velocity of hydrolysis is relatively small then it is practically the same irrespective of whether the enzyme is combined with aluminum hydroxide or not. As a tentative explanation it is suggested that under these conditions the rate of diffusion of the sucrose to and the invert sugar from the enzyme combined with the adsorbent is probably greater than the rate of hydrolysis of the sucrose.

5. No evidence has been obtained so far which indicates that the true activity of the invertase, as distinguished from the resultant rate of hydrolysis of the sucrose in the mixture, is influenced by the combination of the invertase with the adsorbent.

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NEW YORK, N. Y.

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, UNIVERSITY OF PITTSBURGH.]

DERIVATIVES OF 2,4,6-TRINITRO-BENZALDEHYDE.¹ II.

BY ALEXANDER LOWY AND WILMER BALDWIN.

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In the previous report,² the object of the research and references to the literature were given together with the theoretical phase of the subject.

The reactions investigated were all of the general type of condensations previously described³ for the preparation of modified "Schiff's bases."³ Condensations were carried out between 2,4,6-trinitro-benzaldehyde and the following substituted amines: *o*-anisidine, *p*-anisidine, *o*-phenetidine, *p*-phenetidine, *o*-chloro-aniline, *p*-chloro-aniline, and 2,4-dichloro-aniline.

Experimental Part.

Preparation of 2,4,6-Trinitro-benzaldehyde.—The method used in the preparation of this compound was essentially that described by Sachs

¹ This report represents an abstract of a thesis presented by Wilmer Baldwin in partial fulfilment of the requirements for the degree of Master of Science, June, 1921.

² THIS JOURNAL, 43, 341 (1921).

³ Ber., 35, 984 (1902).