with 100 ml. of alcoholic potassium hydroxide. On recrystallization from alcohol, rhombs were obtained, melting at 143° (mixed with γ-fagarine of m. p. 140°, 130°). The yield was 66%.

Anal. Calcd. for $C_{14}H_{13}O_3N$: C, 69.13; H, 5.34; N, 5.76. Found: C, 68.99; H, 5.08; N, 5.79.

Picrate of the Ethoxy Analog of γ-Fagarine.—Obtained from alcohol by the method described, in the form of large yellow prisms, the substance melted at 161°.

Anal. Calcd. for $C_{14}H_{13}O_3N \cdot C_6H_3O_7N_3$: N, 11.86. Found: N, 12.10.

Reconversion of the Ethoxy Analog to γ-Fagarine.—The ethoxy analog of γ-fagarine when treated for two hours with 10% methyl alcoholic potassium hydroxide at 90–100° is converted into γ-fagarine, m. p. 139–141°, showing no depression of m. p. when mixed with the original alkaloid. The picrate melts at 178–179°.

Ethoxy Analog of γ-Fagaric Aldehyde.—A sample of 0.5 g. of the ethoxy analog of fagarine was dissolved in 40 ml. of acetone and slowly treated while warm with 1 gram of potassium permanganate in acetone solution. After the oxidation, the manganese dioxide was filtered off and the filtrate evaporated to dryness. The residue recrystallized from alcohol as yellow needles of m. p. 192–193°. The yield was 228 mg.

Anal. Calcd. for $C_{13}H_{13}O_4N$: C, 63.15; H, 5.26. Found: C, 63.19; H, 5.37.

Phenylhydrazone of the Ethoxy Analog of γ-Fagaric Aldehyde.—Prepared essentially as described, this substance formed yellow plates, melting from 185–186°.

Anal. Calcd. for $C_{19}H_{19}O_3N_3$: N, 12.46. Found: N, 12.64.

Ethoxy Analog of γ-Fagaric Acid.—The manganese dioxide from the previous experiment was extracted with 10% sodium hydroxide. The alkaline solution was boiled for ten minutes, filtered, concentrated, and acidified with dilute hydrochloric acid. The crystalline precipitate thus obtained was recrystallized from acetic acid. Needles melting at 210–211° were obtained: yield, 210 mg.

Anal. Calcd. for $C_{13}H_{13}O_5N$: N, 5.32. Found: N, 5.49.

The same acid is obtained by further oxidation of the ethoxy analog of γ-fagaric aldehyde, m. p. 210–211°.

Methoxy-2,4-dihydroxyquinoline.—A sample of 0.38 g. of the ethoxy analog of γ-fagaric acid was treated with 50 ml. of 30% hydrochloric acid and heated with reflux until total solution. On chilling, crystals came down, which, after recrystallization from 50% alcohol, were small needles, m. p. 250°. The substance obtained from γ-fagarine following the same treatment gave no depression of the melting point, and the nitroso derivatives are also identical.

Summary

1. The action of alkali in alcohol solutions upon skimmianine (β-fagarine) and γ-fagarine has been found to consist of an interchange between the γ methoxyl of these alkaloids and the alkoxyl group of the solvent alcohol.

2. The new alkoxy analogs and several of their derivatives have been described.

3. The reconversion of the new alkoxy analogs into the original alkaloids by action of alkali in methanol has been observed.

4. Possible mechanisms of the changes have been discussed.

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[CONTRIBUTION FROM THE DIVISION OF CHEMISTRY, NATIONAL INSTITUTE OF HEALTH, U. S. PUBLIC HEALTH SERVICE]

New Methods for the Purification of Invertase and Some Properties of the Resulting Products[1]

BY MILDRED ADAMS AND C. S. HUDSON

These investigations on the purification of invertase from yeast were undertaken to develop methods of more general applicability than those reported hitherto. For this purpose we have employed bentonite, previously reported[2] to be an excellent adsorbent for invertase, and several precipitants which we have also found useful in the purification of this enzyme

A critical study has been made of the factors important in obtaining reproducible results in the purification of invertase and our experiments have been carried out with a view to establishing methods as simple as were consistent with maximal purification. As a result, procedures were established which could be used not only for yeast from a constant source, but also for yeast from different sources, and, in spite of marked variations in the invertase content of the starting materials, relatively small differences were noted in the purity of the resulting products. Furthermore, the methods which we have developed are considerably less involved than those generally used, yet the resulting purification was equal to or greater than that produced by the majority of methods reported in the literature.

Our preparations appeared to be protein in

(1) Original manuscript received December 24, 1941.

(2) Adams and Hudson, THIS JOURNAL, **60**, 982 (1938).

nature; they contained more nitrogen than most of the preparations obtained elsewhere and showed the precipitation and color reactions of typical proteins. The most active preparations which we have obtained still contained a small amount of carbohydrate.

Experimental Part

1. Purification by Adsorption

The procedure developed for the use of bentonite as an adsorbent in the purification of invertase consisted of the following steps: autolysis, dialysis, aging, precipitation of inert material at *p*H 3.7–3.9, adsorption on bentonite and elution. Slight variations in the conditions used for each of these steps were essential for the application of the procedure to yeast from different sources[3] and the necessary modifications will be considered in connection with the detailed description of the steps involved.

Autolysis.—Rapid liberation of invertase from the yeast cells was essential for the production of solutions which could be purified readily by adsorption on bentonite. Marked variations in the behavior of yeast from different sources necessitated certain modifications in the procedures generally used for autolysis and the following describes the conditions finally adopted. Enamel containers were used for autolysis in order to avoid any possible effect from contact of the yeast with metal.

Method 1, a slight modification of the method of Hudson,[4a] proved generally satisfactory for brewers' yeast. The yeast was mixed with toluene (10 ml. for each 100 g. of yeast) and allowed to autolyze at 25°. The activity of filtered aliquots was measured daily until the concentration of invertase in solution became constant (three to five days). The autolysate was then filtered with the aid of filtercel (15 g. for each 100 g. of yeast). The addition of water according to the original procedure was omitted, not only to increase the rate of the liberation of invertase, but also to reduce the volume of invertase solution to be treated in subsequent steps of the purification procedure.

Method 2, a different modification,[4a] was necessary for bakers' yeast. The yeast was autolyzed according to the directions for no. 1 except that 50 ml. of water for each 100 g. was added after liquefaction was complete (twenty-four to forty-eight hours); 20 g. of filtercel was added before filtration. The omission of water during the preliminary liquefaction increased markedly the rate of the liberation of invertase, but its subsequent addition was necessary, both for the maximal extraction of invertase, and for the production of autolysates which could be filtered readily. This method gave good results with two bakers' yeasts.

Method 3, a modification of Willstätter's procedure for fractional autolysis,[4b] was necessary for a bakers' yeast which, under the conditions of no. 2, yielded solutions troublesome to purify because of the formation of colloidal suspensions. The yeast was mixed with ether (10 ml. for each 100 g. of yeast) at 30°, and 10 ml. of toluene and 100 ml. of water were added after the yeast had become well liquefied (thirty to sixty minutes). The mixture was allowed to stand for four hours at 25–30°, 20 g. of filtercel was added, and the autolysate was filtered. The filtrate was discarded. The residue was kneaded well with 10 ml. of toluene for each 100 g. of yeast (original weight) and allowed to stand for twenty-four hours at 25–30°; 70 ml. of water was then added. After the liberation of invertase had reached a maximum, usually in three to five days, the autolysate was filtered. Except for one yeast, this method offered no advantages over the simpler methods (1 and 2).

For some of the yeasts investigated, a difference of 5° in the temperature during autolysis was sufficient to influence appreciably the resulting bentonite purification, although for others as much as a 15° variation was possible. In spite of such differences, a temperature of 25° proved satisfactory for the five yeasts autolyzed by methods 1 and 2. For method 3, a temperature of 30° was necessary for the first fractionation and 25–30° proved satisfactory for the second. The following examples indicate the type of variation noted. From one brewers' yeast, preparations were obtained with time values[5] 0.17, 0.17 and 0.20 minute when autolysis was carried out at 20, 25 and 30°, respectively; from another, preparations with time values of 0.19, 0.16, 0.16 and 0.16 minute correspond to autolysis at 20, 25, 30 and 35°, respectively.

The *p*H of the autolysates, prepared as directed, varied from 5.0–6.7. No correlation was noted, however, between these variations in *p*H and the modifications found necessary in applying the purification procedures to yeast from different sources.

Autolysis, direct or fractional, in the presence of sodium carbonate[6] produced invertase solutions from which preparations have been obtained comparable in purity to those from autolysates prepared by methods 1–3, but, occasion-

(3) Brewers' bottom fermentation yeast was supplied through the courtesy of the Christian Heurich Brewing Co., Washington, D. C., the Gunther Brewing Co., Baltimore, Md., and the National Brewing Co., Baltimore, Md. The yeast was filtered the day it was received from the brewery and autolysis started immediately after filtration. Starch-free compressed bakers' yeast (freshly compressed if available or compressed immediately prior to shipment) was purchased from Standard Brands, Inc., Langdon, D. C., the Compressed Yeast Corporation of America, New York City, and the Red Star Yeast and Products Co., Milwaukee, Wis.

(4) (a) Hudson, THIS JOURNAL, **36**, 1566 (1914); (b) Willstätter, Schneider and Bamann, *Z. physiol. Chem.*, **147**, 248 (1925).

(5) The time value, a term proposed by O'Sullivan and Tompson [*J. Chem. Soc.*, **57**, 834 (1890)] is defined (C. Oppenheimer, "Die Fermente und ihre Wirkungen," Verlag Georg Thieme, Leipzig, fifth edition, 1929, Vol. III, p. 770) as the time in minutes required at 15.5° for 0.05 g. of invertase preparation to invert to 0° rotation (referred to the D line of sodium light) 4.0 g. of sucrose in 25 ml. of solution at *p*H 4.5. For convenience, our hydrolysis measurements were made at 20°, and the values thus obtained were converted to time values (at 15.5°) by multiplying by 1.33, a factor determined experimentally and found to be constant for untreated autolysates as well as for purified preparations.

(6) Standard Brands, Inc., British Patent 418,211, Oct. 22, 1934.

ally, difficulties were encountered in filtering such autolysates and in obtaining reproducible results.

Dialysis.—Dialysis proved very important in the purification procedures and all attempts to eliminate it failed to yield the highly purified products obtained when it was included. In addition to the removal of dialyzable material, this process effected fundamental changes in the properties of the resulting invertase solutions. The conditions maintained during dialysis must be controlled carefully. Certain treatments preliminary to dialysis, used by numerous investigators for other methods of purification, proved to be undesirable for the best results with the methods developed here. The following describes the necessary precautions and the conditions of dialysis finally adopted.

(a) For all of the yeasts investigated, immediate dialysis, after filtration of the autolysate, was of utmost importance. The introduction, prior to dialysis, of an aging period, of acid precipitation to remove inert material, or of acetone or alcohol precipitation of invertase, to separate it from some of its accompanying impurities, decreased appreciably the ultimate purification.

Table I illustrates the results obtained when two such procedures were used. Alcohol or acetone precipitation of invertase before dialysis proved even less desirable. The data in this table also indicate the importance of evaluating results on the basis of the time value of the extract from bentonite rather than on that of the invertase solution resulting from any particular treatment. Throughout this report, therefore, the effectiveness of the various modifications studied has been determined on the basis of the time values of the bentonite-purified products obtained under the optimal conditions for each modification used.

TABLE I

COMPARISON OF THE PURIFICATION OF INVERTASE RESULTING FROM UNTREATED DIALYZED SOLUTIONS WITH THAT FROM SOLUTIONS AGED OR TREATED WITH ACID PRIOR TO DIALYSIS

Starting material	Treatment prior to dialysis	Dialyzed autolysate, time value, min.	Extract from bentonite[a] Extraction, %	Extract from bentonite[a] Time value, min.
Brewers' Yeast 1, time value 149 min.	None	11.0	(1) 74	0.15
			(2) 16	.16
	Aged one month	7.0	(1) 83	.19
			(2) 11	.22
	Adjusted to *p*H 4.5 with glacial acetic acid and filtered	6.8	(1) 75	.19
			(2) 12	.23
Bakers' Yeast 2, time value 38 min.	None	3.1	(1) 63	.18
			(2) 9	.20
	Aged one month	Unable to obtain solutions suitable for use with bentonite		
	Adjusted to *p*H 4.5 with glacial acetic acid and filtered	3.0	(1) 61	0.32
			Difficulty with colloidal suspensions	

[a] The bentonite was extracted twice and the extracts were kept separate because of the consistently higher time values observed for the second extracts.

(b) The addition of toluene or of thymol to the filtered autolysate prior to dialysis should be avoided. The influence of these preservatives varied somewhat with different autolysates, but, in general, excessive amounts of bentonite were required for the adsorption of invertase from solutions dialyzed in their presence and purified products were obtained with time values as high as 0.45 minute. These preservatives exerted little influence on the activity of invertase itself, but prevented changes necessary for the production of solutions suitable for purification by means of bentonite, possibly by influencing the activity of other enzymes responsible for the desired results.

(c) Dialysis, using Visking sausage casings[7] as membranes, was carried out against running tap water (three to four liters per minute), at 28–30°, with two to three liters of the solution to be dialyzed in thirty-six liter enamel containers. The temperature of 28–30° was adopted in view of the results obtained when the temperature during dialysis was varied from 5–37°. Consistent increases in adsorption and purification were noted with increasing temperatures up to 30° and no further improvement resulted from dialysis at 37°. Prolonged dialysis at 5° gave solutions from which invertase was adsorbed poorly by bentonite and subsequent dialysis at room temperature failed to improve materially the adsorption of this enzyme.

No appreciable differences in purification have been observed as the result of varying the rate of flow during dialysis from two to four liters per minute, but decreased purification resulted from solutions dialyzed at a rate slower than this. In order to allow some margin of safety, therefore, dialysis against tap water flowing at a rate of three to four liters per minute has been selected as a standard condition.

(d) Although the optimal length of time for dialysis was practically constant for invertase solutions from different lots of the same yeast, variations in this respect, with solutions from different yeasts, necessitated establishing this period carefully for each new starting material by ascertaining the purification resulting from aliquots dialyzed for varying periods of time (twelve-hour intervals).

Invertase solutions resulting from too short a period of dialysis were poorly adsorbed and required more acid solutions for adsorption than were generally necessary; the recovery of invertase in the eluate from bentonite was low as well as the ultimate purification. On the other hand, if dialysis was carried out for too long a period, considerable difficulty was encountered with colloidal suspensions; appreciable loss of activity occurred during the subsequent treatment with acetic acid; less highly purified preparations generally resulted. The following periods of dialysis were found suitable for the six yeasts studied: brewers' 1 and 3, forty-eight hours; brewers' 2, sixty hours; bakers' 1 and 2, twenty-four hours; bakers' 3, thirty-six hours. No correlation was observed between the time value of the starting material and the optimal duration of dialysis.

Aging.—If dialyzed autolysates were allowed to stand for one to two weeks before further treatment, the amount of bentonite required for adsorption was considerably decreased and the final purification improved. Moreover,

(7) Visking Corporation, Chicago, Ill.

the difference between the time values of the first and the second extracts was generally reduced.

Although this optimal period was constant for different lots of the same yeast, it was necessary to establish this period for each new yeast by determining the purification resulting from the use of aliquots of the dialyzed autolysate which have aged for one and two weeks, respectively. Solutions aged for less than one week were unsatisfactory because the amount of bentonite necessary for the adsorption of invertase was large and changed rapidly during the first few days after dialysis. For five of the yeasts investigated, more consistent results were obtained after aging for two weeks, but for the sixth yeast, a period of one week proved distinctly better than the longer period. Aging for a period of more than two weeks was generally unnecessary, and in many cases definitely undesirable.

The following gives an example of the influence of aging a dialyzed autolysate from brewers' yeast on the adsorption and purification of invertase: seven days after dialysis, 1 g. of bentonite adsorbed 24 units[8] of invertase and extracts with time values of 0.17 and 0.21 minute were obtained; after fourteen days, 1 g. of bentonite adsorbed 36 units and extracts with time values of 0.15 and 0.16 minute resulted.

Toluene could be added for the preservation of the dialyzed autolysates during the required aging period; certain necessary precautions will be considered in detail in the next section.

Acid Precipitation of Inert Material.—The precipitation of inert material with acid, although undesirable before dialysis, proved essential prior to adsorption of invertase on bentonite. In addition to the removal of inert material, this treatment facilitated the separation of the precipitate formed during dialysis and improved the adsorption of invertase from many of the solutions from brewers' yeast.

After aging, the dialyzed autolysate was adjusted to *p*H 3.7–3.9 by the addition of *N* acetic acid, allowed to stand for one hour, centrifuged to remove the bulk of the precipitate, and filtered through filter paper precoated with a thin layer of filtercel to remove suspended material not separated by centrifugation. Excess filtercel must be avoided because of its ability to adsorb invertase somewhat at this stage in the procedure.

Occasionally solutions were encountered which, after acidification as described, gave filtrates with negligible invertase activity. This acid precipitation of invertase occurred in solutions from brewers' and from bakers' yeast, although much more frequently in the latter. As the result of changes produced by the insoluble material present in these solutions after dialysis, the precipitability of invertase disappeared, generally within forty-eight hours, and such solutions could be purified by the usual procedure with bentonite. If toluene was added as a preservative, however, invertase continued to precipitate in acid for a much longer period of time. As a general rule, therefore, aliquots of each autolysate were tested for acid precipitability of invertase immediately after dialysis, and daily thereafter, until invertase no longer precipitated. Toluene was then added and the solutions aged as usual before the final acid treatment.

(8) One unit of invertase will invert 4.0 g. of sucrose to 0° rotation in one minute under the standard conditions described in footnote 5.

Adsorption.—Wyoming bentonite[9] was used as an adsorbent throughout these investigations. In spite of careful control of the factors found to be important during the preliminary stages of the purification procedure, certain relatively simple tests were necessary before the final adsorption could be carried out.

In the first place, it was necessary to determine whether the acid-treated invertase solutions could be purified by adsorption on bentonite immediately, or whether such solutions needed to stand for a short period prior to adsorption. The following example indicates how this point was established. Thirteen days after dialysis of an autolysate from brewers' yeast, a 75-ml. portion was treated with 22.5 ml. of *N* acetic acid and the precipitate removed as previously described. On the following day, the remaining solution was treated with acid in a similar way, A 1.5-ml. portion of a 0.25% suspension of bentonite was then added to a 5-ml. aliquot of each of these solutions and measurements were made of the activity remaining in the supernatant solutions after centrifugation. Bentonite removed 89.6% of the invertase in the aliquot treated with acid on the thirteenth day and 23.5% from the solution so treated on the fourteenth day.

When such differences in adsorption were noted the two solutions which had been treated with acid were allowed to stand an additional twenty-four hours in this acid condition and adsorption determined again. The addition of 1.4 ml.[10] of a 0.25% suspension of bentonite to a 5-ml. aliquot of the solution which had been acidified on the thirteenth day and kept in this acidity forty-eight hours resulted in adsorption of 84.7% of the invertase present; a similar addition to an aliquot of the solution which had been acidified on the fourteenth day and kept in this acidity twenty-four hours caused 82.5% adsorption. For this particular solution, therefore, twenty-four hours of standing in acid was sufficient and the solution acidified on the fourteenth day was ready for the final purification.

If, however, marked differences in adsorption were still noted (more than 15%) daily measurements of adsorption were continued as described until these differences did not exceed 15%. It was important to purify these solutions as soon as possible after this approximately maximal adsorption occurred, because twenty-four hours in acid, in excess of that necessary, was often sufficient to reduce materially the ultimate purification.

Most solutions from brewers' yeast required at least a twenty-four-hour period in acid before carrying out the final adsorption on bentonite. Most solutions from bakers' yeast, on the other hand, did not need to stand in acid before the final bentonite purification.

In the second place, it was necessary to determine the optimal *p*H for adsorption. Figure 1 indicates the marked influence of slight changes in acidity on the adsorption of

(9) Five different lots of bentonite have been used and yielded preparations with time values agreeing within 0.01 minute. The bentonite samples were kindly supplied by The American Colloid Co., Chicago, Ill. (200 mesh Volclay and KWK Volclay); Harry Haze, Inc., Chicago, Ill. (Owyhee Wilkinite); George F. Pettinos, Inc., Philadelphia, Pa.; Wishnick-Tumpeer, Inc., New York (Witco bentonite, 325 mesh).

(10) The amount of bentonite used was reduced to permit satisfactory measurement in the case of further improvement in adsorption. Occasionally it was necessary to repeat with still smaller amounts of bentonite.

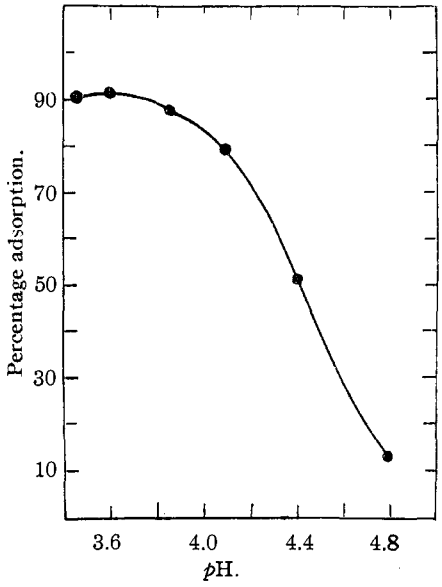

Fig. 1.—Typical curve showing the influence of *p*H on the adsorption of invertase by bentonite.

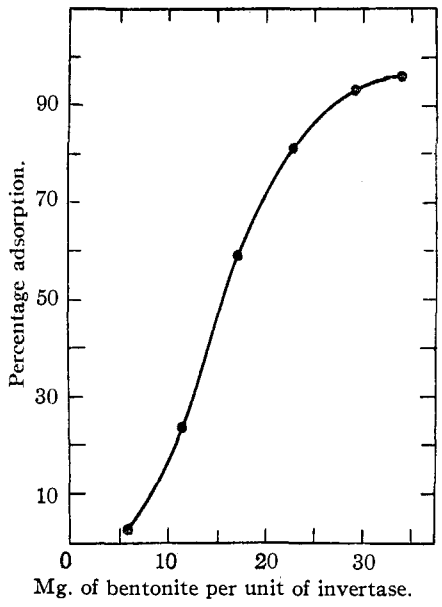

Fig. 2.—Typical curve showing the influence of the concentration of bentonite on the adsorption of invertase.

invertase on bentonite. The best purification was obtained when invertase was adsorbed at the highest *p*H which was consistent with maximal adsorption. Because of variations in this optimum (*p*H 3.6–4.1) for different invertase solutions, it was necessary, after aging in acid for the requisite period of time, to establish the conditions suitable for each new solution by determining the adsorption in 5-ml. aliquots adjusted to *p*H 3.6, 3.8, 4.0 and 4.2.

Finally the amount of bentonite necessary to adsorb 90–97% of the invertase in solution was determined by measuring the adsorption which resulted from the treatment of 5-ml. aliquots with varying amounts of bentonite at the proper *p*H (see Fig. 2).

Sufficient information was then available for proceeding with the final purification. In all cases, adsorption occurred rapidly and the suspensions were centrifuged about ten minutes after the addition of bentonite.

Fractional adsorption based upon a preliminary treatment with bentonite under the conditions removing 10–20% of the invertase effected about the same purification (time value, average 0.152 minute for five different lots of yeast) as that (average, 0.157 minute) resulting from the simpler methods described.

Elution.—The invertase was removed readily from the bentonite by shaking it gently for about two minutes with an acetate solution adjusted to *p*H 5.4–5.6 (a mixture of 0.3 *N* acetic acid and 0.3 *N* sodium hydroxide). The suspension was then allowed to settle for a few minutes, centrifuged, and the extract filtered through filtercel to remove any remaining particles of bentonite. The time values were determined by measuring the activity and the solids present in these extracts after dialysis for one week against distilled water at 5°.

Because of the relatively small amount of adsorbent necessary per unit of invertase, it was possible to extract with a small volume of eluent and thus to obtain more concentrated solutions than have generally been prepared by methods developed elsewhere. The following procedure was adopted: 100 ml. of eluent was used for each

TABLE II

EXAMPLE OF THE PROCEDURE FOR THE BENTONITE PURIFICATION OF INVERTASE

I. *Autolysis:* 2200 g. of brewers' yeast (time value, 138 minutes) + 220 ml. toluene, (25°, 4 days); filtrate 1340 ml.
Recovery, 82.7% Time value, 77 minutes

II. *Dialysis:* 1300 ml. filtrate dialyzed immediately against running tap water, 28–30°, 48 hours; dialyzed autolysate, 1740 ml.
Recovery, 99.8% Time value, 11.4 minutes

III. *Aging:* dialyzed solution aged two weeks at room temperature, with toluene added.

IV. *Acid precipitation of inert material:* 1600 ml. of aged solution + 480 ml. *N* acetic acid; filtrate 2030 ml., *p*H 3.8.
Recovery, 92.0%

V. *Adsorption:* acid filtrate allowed to stand twenty-four hours; 1880 ml. + 188 ml. *N* acetic acid + 602 ml. 0.25% suspension of bentonite; centrifuged; 2620 ml., *p*H 3.7; residue washed once with 1000 nil. water by centrifugation.
Adsorption, 95.3%

VI. *Elution:* residue extracted with buffer solution, *p*H 5.4 (0.3 *N* acetic acid and 0.3 *N* NaOH); centrifuged; filtered through filtercel.
Extract 1, 184 ml.
Recovery, 82.9% Time value, 0.161 minute[a]
Extract 2, 102 ml.
Recovery, 12.6% Time value, 0.195 minute[a]
Total recovery in two extracts referred to original activity of the yeast 72.5%.

[a] Based on measurements of the activity and solids in aliquots dialyzed for one week at 5°.

25 units of adsorbed invertase, and 60–80% of the invertase was liberated; a second extraction with 50 ml. liberated an additional 10–20%. A reduction of the volume of eluent below the recommended amount decreased appreciably the yield of invertase.

An example of the entire procedure as applied to brewers' yeast is presented in Table II.

The low temperatures reported by numerous investigators as desirable when kaolin was used as an adsorbent were not necessary for bentonite; therefore, the various steps in the procedure have been carried out at room temperature except where otherwise indicated. The yield of invertase differed somewhat with different lots of yeast, but rarely was below 60%. This was due chiefly to variations in the liberation of invertase during autolysis, both from brewers' yeast and from bakers' yeast, and to somewhat poorer extraction from bentonite when bakers' yeast was used as a starting material.

Table III summarizes the results of applying the method as finally developed to different lots of the same yeast and to yeast from six different sources. In addition to data obtained for the four yeasts most extensively investigated, results have also been included to indicate the purification resulting in the case of (a) two yeasts studied to determine the general applicability of the method to new sources of invertase, (b) two yeasts of very low invertase content, and (c) two enriched brewers' yeasts.

The results indicate the possibility of obtaining invertase solutions differing very little in their time values, in spite of

TABLE III

COMPARISON OF THE BENTONITE PURIFICATION OF INVERTASE RESULTING FROM SOLUTIONS OBTAINED FROM YEAST FROM SIX DIFFERENT SOURCES

Source	Yeast: No. of samples	Yeast: Time value, min., range	Purified preparations: Time value, min. Range	Purified preparations: Time value, min. Average
Brewers' 1	8	136–208	0.150–0.167	0.160
Brewers' 2	7	117–272	.158–0.176	.166
Brewers' 3	1	275	.168	
Brewers' 1[a]	1	442	.167	
Enriched Brewers' 1[b]	1	63	.153	
Enriched Brewers' 2[b]	1	67	.150	
Bakers' 1	6	23–28	.181–0.203	.190
Bakers' 2	8	28–44	.181–0.233	.191
Bakers' 3 grown on grain	2	52–62	.171–0.179	
Bakers' 1[c]	1	414	.197	

[a] Yeast of this time value was not available for use under the conditions finally adopted but the results given indicate the possibility of obtaining highly purified preparations from yeast of very low invertase content. [b] The invertase content of this yeast was increased by the use of conditions essentially as recommended by Weidenhagen [*Z. angew. Chem.*, **47**, 581 (1934)]. [c] During the early stages of our investigations the invertase content of the several bakers' yeasts studied was somewhat lower than that observed later, and occasionally yeast was obtained which had an exceptionally high time value such as the one cited above. No explanation was apparent.

marked variations in the time values of the starting materials. In general, the preparations from brewers' yeast were of somewhat lower time value than those from bakers' yeast. However, the high invertase content of the bakers' yeasts made it possible to produce about six times as much purified invertase as from an equal weight of the brewers' yeast.

Under the conditions finally adopted, colloidal solutions caused no trouble in the purification of invertase from brewers' yeast, and only occasionally with solutions from bakers' yeast.

A second adsorption with bentonite produced little additional purification and the yields of invertase obtained were generally low. Likewise, alumina, another adsorbent used extensively by other investigators, failed to improve appreciably the time values of the bentonite-purified products.

2. Purification by Precipitation

Acid and Base Precipitants.—In the course of our investigations, we have noted that invertase, in solutions prepared under the conditions of autolysis and dialysis which we have recommended, behaved as an ampholyte, precipitating either with acid or base precipitants. Methods have been developed, therefore, for the purification of invertase by precipitation with picric acid, flavianic acid, Reinecke salt, ammonium rhodanilate and picrolonic acid (base precipitants); cupric acetate and uranyl acetate (acid precipitants).

The following summarizes the precautions necessary and the conditions established for the use of these reagents in the production of highly purified preparations.

(a) For purification by precipitation, invertase solutions were prepared by the same preliminary procedures[11] (autolysis, dialysis, aging and acid precipitation) as have already been described for purification by adsorption, except that the short aging period subsequent to acetic acid precipitation was unnecessary, even for solutions from brewers' yeast.

(b) The optimal *p*H for precipitation varied in different invertase solutions from 3.7–4.5 with base precipitants and from 4.7–6.0 with acid precipitants. Variations were observed also in the minimal amount of reagent necessary for precipitation. It was necessary, therefore, to establish the optimal conditions by preliminary tests on aliquots of each invertase solution. Denaturation and inactivation were prevented by keeping all solutions ice-cold during precipitation and by avoiding exposure of

(11) Invertase did not exhibit amphoteric properties in solutions prepared under conditions previously found to be unsuitable for the best results with bentonite. This enzyme failed to precipitate with base precipitants from autolysates treated with acetone before dialysis or dialyzed at 5° and also, from most autolysates aged or precipitated with acid prior to dialysis. Preparations have been obtained by the use of acid precipitants in such solutions but the resulting products were of relatively high time values.

invertase to excess acid.[12] Two hours after addition of the reagent to the invertase solution, the resulting precipitate was separated, as rapidly as possible, by filtration through filtercel and washed with an ice-cold solution of the reagent (prepared by mixing reagent and water in the same proportions as were used for reagent and invertase solution in the precipitation, and by adjusting the *p*H to that used for precipitation).

(c) For extraction, the filter paper, filtercel and precipitate were transferred to a beaker, mixed well with acetate buffer (*p*H 5.4–5.6 for precipitates obtained with base precipitants; 3.7–4.4 for those obtained with acid precipitants) and the resulting extract filtered after a period of thirty minutes.

(d) The precipitant was removed by dialysis, first against an acetate solution of the same *p*H as that used for extraction until no perceptible color due to the reagent remained, and finally against distilled water.

Typical data indicating the results obtained with picric acid are recorded in Table IV. In general, picric acid proved somewhat less effective than bentonite in the purification of invertase when used directly in the acid-treated, dialyzed autolysates from brewers' yeast. If, however, invertase was first precipitated from such autolysates by acetone (70–100 ml. for each 100 ml. of autolysate) and the aqueous extract[13] of this precipitate (extraction with a volume approximately one-fifth that of the original volume) treated with picric acid, the time values of the resulting products were equal to or slightly lower than those of the corresponding bentonite-purified preparations. This preliminary precipitation by acetone was unnecessary for the solutions of high invertase content from bakers' yeast.

When the time values of the bentonite-purified products from brewers' yeast were higher than 0.15 minute, or from bakers' yeast higher than 0.18 minute, a slight additional purification generally resulted from the use of picric acid. However, the maximal purification resulting from the use of this reagent, with one exception, was identical with that obtained by the use of bentonite.

One preparation with a time value of 0.140 minute (Table III, 5) was obtained by filtering the extract immediately instead of allowing the usual thirty-minute period for extraction. This reduction of the extraction period

(12) In the case of Reinecke salt, ammonium rhodanilate, cupric acetate, or uranyl acetate, this was accomplished readily by adjusting the invertase solution, with acetate buffer, to the *p*H necessary for precipitation before the addition of reagent. Because picrolonic, picric and flavianic acids exerted an appreciable influence on the *p*H of invertase solutions adjusted as described, a slightly different procedure was necessary for use with these reagents. In the case of picrolonic acid, invertase solutions were adjusted to *p*H 4.5–4.7, somewhat higher than desired for precipitation, and acetic acid was added, if necessary, following the addition of this reagent. In the case of picric or flavianic acid, it was necessary to adjust the solution of the reagents to *p*H 4.5–4.7 and the mixture of enzyme and precipitant was finally brought to the desired *p*H by the cautious addition of *N* acetic acid. Aqueous solutions of the reagents were used in all cases.

(13) Invertase was readily adsorbed by bentonite and precipitated by picric acid from solutions prepared in this way, whereas adsorption was poor and no precipitation occurred from autolysates treated with acetone before dialysis. These results suggest that the changes in the properties of invertase as the result of dialysis under the conditions which we have adopted were due to some factor, possibly enzymic in nature, which was separated when precipitation by acetone was carried out prior to dialysis.

TABLE IV

THE PURIFICATION OF INVERTASE BY PRECIPITATION WITH PICRIC ACID

	Description of starting material	Picric acid-purified product: % Recovery[a]	Picric acid-purified product: Time value, min.	Bentonite-purified product[b]: Time value, min.
1	Brewers' yeast, dialyzed autolysate, time value 18.4 min.	88	0.206	0.161
2	Bakers' yeast, dialyzed autolysate, time value 3.5 min.	78	.185	.186
3	Brewers' yeast, dialyzed autolysate, time value 17.1 min., acetone precipitated, time value 8.82 min.	65	.150	.174
4	Brewers' yeast, bentonite-purified, time value 0.173 min.	70	.155	
5	Brewers' yeast, bentonite-purified, time value 0.153 min.	45	.140	
		20	.152	

[a] Recovery referred to activity of solution from which invertase was precipitated with picric acid. [b] These figures represent the purification resulting from the use of bentonite in an aliquot of the corresponding dialyzed autolysate described in the first column.

apparently prevented the solution of a small amount of inert material which dissolved under the usual conditions but, at the same time, decreased considerably the yield of invertase.

Attempts to effect still further purification by fractional precipitation with picric acid were unsuccessful.

Picric acid, in addition to its value in the purification of invertase, also provided a means of preparing highly concentrated solutions of this enzyme which contained as much as 230 units of invertase in 100 ml. These solutions could be obtained more rapidly than by the usual methods of concentration and with relatively small losses in invertase activity.

Picric acid proved to be the most useful of the precipitants studied and therefore has been the most extensively investigated. Highly purified preparations of invertase have been obtained by the use of each of the other base precipitants investigated but, in many cases, particularly when used directly in dialyzed autolysates, these reagents have proved to be slightly less effective than picric acid in the purification of this enzyme. The acid precipitants[14] were not as satisfactory as the base precipitants because, in most cases, less purification was produced and significant losses in activity were observed upon dialyzing some of the resulting products.

Still another method for the purification of invertase was available for those dialyzed autolysates from which, as previously described, invertase precipitated upon acidification to *p*H 3.7–4.2. By means of a series of such precipitations, followed in each case by extraction with acetate buffer of *p*H 5.4, invertase preparations have been obtained from brewers' yeast with time values of 0.17 minute, and from bakers' yeast with time values of 0.22 minute. Because of the influence of aging on the pre-

(14) Willstätter and Racke [*Ann.*, **425**, 1 (1921)] and Weidenhagen and Neuninger [*Z. Wirtschaftsgruppe Zuckerind.*, **89**, 149 (1939)] have used uranyl acetate for the purification of invertase but the time values of the products obtained under the conditions which they have employed were higher than those of our preparations.

TABLE V

COMPARISON OF THE CHEMICAL COMPOSITION OF DIFFERENT INVERTASE PREPARATIONS

	Purification procedures	Time value, min	N, %	P, %	Reducing material calculated as glucose, % Total	Fermentable	Units of invertase per mg. N
1	Bentonite adsorption, followed by picric acid precipitation	0.140	14.8	0.03	6.9[a]	3.2[b]	0.96
2	Prolonged aging before and after dialysis; bentonite adsorption, followed by acetone precipitation	0.224	9.9	0.12	29.2[a]	25.1[b]	0.90
3	Preparation reported by Weidenhagen (ref. 16); enriched yeast, strontium hydroxide precipitation of autolysate, then uranyl acetate precipitation, followed by alcohol precipitation	(0.296)[c]	7.5		48.8		0.90

[a] Determined by method of Shaffer and Hartmann [*J. Biol. Chem.*, **45**, 349 (1921)] in solutions previously hydrolyzed for four hours in the presence of hydrochloric acid (8 ml. of concd. hydrochloric acid per 100 ml. solution). These figures obtained from determinations in the nitrogen-containing filtrate probably represent some non-carbohydrate reducing material. [b] These figures represent the carbohydrate actually fermented by yeast. [c] Calculated by dividing 166 by the fructosidase value, 560.

cipitation in this way, it was necessary to carry out this procedure as soon as possible after dialysis was completed.

In view of the phosphorus content of these preparations (about 1% of their dry weight in contrast to 0.03% in the bentonite-purified products), the direct correlation between the phosphorus content and the precipitability of invertase, and the redispersion of these precipitates by the addition of a small amount of a salt, it seemed likely that, in this method of purification, we were dealing with a coacervate of invertase with nucleic acid present in these autolysates immediately after dialysis. Further evidence of this was seen in the production of similar preparations of invertase by precipitating this enzyme with nucleic acid at *p*H 3.7–4.2 from solutions which failed to respond to acid treatment in the manner just described.

The activity of these preparations on the basis of the solids obtained by difference from the total solids and nucleic acid (calculated from the phosphorus content) was practically the same as that of the products obtained by the use of bentonite or of picric acid.

Although certain factors (temperatures of 30–35° during autolysis, a temperature of 37° or the presence of toluene during dialysis) seemed to favor the production of solutions containing invertase which precipitated upon acidification as described, we have not been successful in establishing conditions which permitted the consistent preparation of such solutions.

Ammonium Sulfate Precipitation.—Considerable variation has been reported in the literature with respect to the solubility of different invertase preparations in saturated ammonium sulfate solutions. As the result of our investigations, we have found that the factors important in the preparation of invertase solutions suitable for purification by the methods which we have already described, favored also the production of solutions from which invertase was precipitated by saturation with ammonium sulfate. Furthermore, ammonium sulfate proved about as effective in the purification of invertase as the other methods which we have considered previously. For example, by the addition of a saturated ammonium sulfate solution (200 ml.[15] for each 100 ml. of invertase solution) to a dialyzed autolysate from bakers' yeast (time value, 1.64 minutes), a preparation was obtained with a time value of 0.215 minute; in a similar way, from a bentonite-purified product (time value, 0.164 minute) a preparation was obtained with a time value of 0.150 minute.

In the case of ammonium sulfate we are dealing with a salting out of the invertase due to dehydration rather than with a precipitate, the formation of which is dependent upon the ionization of invertase as an acid or as a base. Although it was possible, therefore, to precipitate invertase with ammonium sulfate from solutions varying from *p*H 3.8–6.0, less salt was necessary for and occasionally higher purification resulted from precipitation at *p*H 3.8–4.4 than at a higher *p*H.

No further purification was obtained by fractional precipitation of invertase with ammonium sulfate.

3. Properties of the Purified Preparations

Invertase preparations obtained by the methods which we have developed appeared to consist chiefly of protein. They contained more nitrogen, on the basis of their dry weight, than the majority of purified products prepared elsewhere. They were coagulated upon heating and were precipitated by the usual protein precipitants. They were insoluble in ammonium sulfate solutions (50–70% saturated). Inactivation always accompanied denaturation. The biuret, tryptophan, Millon and ninhydrin reactions were all positive. The Molisch test for carbohydrate was positive, and the Salkowski test for yeast gums, negative. Data obtained from analysis of a preparation, no. 1, which gave the qualitative tests just described, are recorded in Table V.

A preparation obtained by the purification of

(15) The amount of ammonium sulfate necessary for precipitation varied somewhat with the *p*H and with the concentration of the invertase solution.

an autolysate which was aged before dialysis (a procedure not recommended for the best results with bentonite) was found to exhibit properties differing considerably from those of our usual preparations. This preparation was not coagulated upon heating and failed to give the usual protein precipitation tests. It failed to precipitate in 50–70% saturated ammonium sulfate solutions, but 60% of the invertase present in this preparation was precipitated by complete saturation with this salt. Fractionation in this way, however, failed to produce, either from the filtrate or from the precipitate, a product of higher purity than the original solution. The biuret, tryptophan, Millon and ninhydrin reactions (also given by protein degradation products) were all positive. Data obtained from analysis of this preparation, no. 2, are also reported in Table V. In addition, we have included for comparative purposes the data which Weidenhagen[16] has reported for a preparation with properties,[17] in general, similar to those which we have observed for no. 2.

The most active of these preparations, 1, contained the smallest amount of carbohydrate. Although it is impossible to say, from our present information, whether or not the small amount of carbohydrate in 1 is essential, it is obvious that much of the carbohydrate in 2 and 3 is not essential to the activity of this enzyme.

The results reported in Table V indicate that the conditions which we have established for the purification of invertase favor the separation of this enzyme from a large amount of the carbohydrate originally accompanying it. These results suggested also that the large amount of carbohydrate present in 2 and 3 is responsible for the failure of these preparations to respond to the usual protein precipitation tests.[18]

Although contradictory results have been reported[19] with regard to the proportionality between activity and nitrogen content of different invertase preparations, a striking parallelism is noted in this respect for the three preparations described in Table V, in spite of marked differences in their time values, their properties and the methods used for their preparation.

The invertase solutions prepared by the methods which we have described lost no appreciable activity over a period of several months when stored at 5°. They were stable to dialysis at 5° and even when dialyzed at room temperature lost relatively little activity. Dry products have been obtained by means of precipitation with acetone or by dehydration from the frozen state. Such preparations, however, were generally of higher time values than the solutions from which they were prepared, because of the tendency for invertase to become denatured in the process. Invertase proved to be very soluble in aqueous solutions. By concentrating a product *in vacuo*, at room temperature, we have obtained one preparation containing 9.6 g. of solids and 940 units of invertase in 100 ml. of solution.

A constant maximal purification resulted irrespective of whether invertase was purified by adsorption on bentonite, by precipitation with picric acid or with ammonium sulfate, or by a combination of these procedures. These results indicate that our preparations are either homogeneous or a mixture of substances, apparently protein, so closely related chemically that no separation has been possible over a wide range of conditions. Furthermore, preliminary attempts to produce additional purification by means of ultracentrifugation or ultrafiltration have been unsuccessful. The difficulties in establishing the purity of a protein[20] are well known, however, and further evidence, such as can be obtained by means of solubility measurements and electrophoresis, is necessary to determine whether or not these preparations are homogeneous. The methods which we have developed permit the preparation of highly active invertase solutions in sufficiently large amounts to make such studies possible.

Although our preparations of invertase were of

(16) Weidenhagen and Neuninger, *Z. Wirtschaftsgruppe Zuckerind.*, **89**, 149 (1939).

(17) These investigators state that solutions of their enzyme gave no precipitate either in solutions half saturated with ammonium or magnesium sulfate or in solutions saturated to a still higher degree. It is possible, therefore, that preparation 3 differed somewhat from 2 by being completely soluble even in saturated ammonium sulfate solutions.

(18) Lutz and Nelson [*J. Biol. Chem.*, **107**, 169 (1934)] have reported the preparation of invertase solutions which failed to respond to the usual protein precipitation tests, and in general behaved qualitatively like preparations 2 and 3. These preparations apparently contained protein, however, because the dry enzyme preparation and its aqueous solutions showed good protein and yeast gum tests [Nelson, cited by Tauber, "Enzyme Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1937, p. 127].

(19) Euler and Josephson [*Ber.*, **56**, 1097 (1923)] have indicated for their preparations a correlation between invertase activity and nitrogen which did not differ markedly from that which we have observed, whereas Willstätter and Schneider [*Z. physiol. Chem.*, **133**, 193 (1924)] failed to observe a consistent proportionality in this respect for their preparations.

(20) Pirie, *Biol. Rev.*, **15**, 377 (1940).

lower time values than those of the majority of purified preparations obtained elsewhere, a few products of exceptionally low time value have been reported. Lutz and Nelson[18] have obtained preparations with time values of 0.10–0.11 minute and Willstätter and his co-workers[21] have reported occasional preparations with similar time values. The still lower time values reported for a few other preparations[22] are questionable because of the instability of these products to dialysis.

These preparations of exceptionally low time values were obtained by involved procedures which have not proved to be of general applicability.[23] Their production may be the result, either of the removal of inert material not separated by the various procedures which we have used, or of an actual modification of invertase itself produced by the method used for its purification. Unfortunately, insufficient data are available for adequate comparison of these preparations with those obtained in this Laboratory.

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Summary

A method has been described for the purification of invertase by the use of bentonite as an adsorbent; with slight modifications this method has proved applicable to yeast from six different sources. The method is relatively simple and as effective as more involved procedures which include enrichment of the yeast or fractional adsorption. Preparations of practically constant time values were produced, in spite of a nineteenfold variation in the invertase content of the starting materials.

In solutions prepared under the conditions which we have described, the invertase exhibited amphoteric properties which made possible its purification by precipitation with both acid and base precipitants. Methods have been developed for the purification of this enzyme by the use of picric, picrolonic, flavianic and nucleic acids, Reinecke salt, ammonium rhodanilate, cupric acetate and uranyl acetate. In general, the base precipitants proved more satisfactory than the acid precipitants and the maximal purification produced by these reagents was approximately the same as that resulting from the use of bentonite. These precipitants proved particularly useful when highly concentrated solutions of invertase were desired.

Purified products similar to those obtained by adsorption or by precipitation have resulted from the use of a third type of procedure based upon the insolubility of invertase in saturated ammonium sulfate solutions.

Qualitative and quantitative tests have been reported indicating that our preparations consisted chiefly of protein although they still contained a small amount of carbohydrate.

Results have been described suggesting that the contradictory reports, with respect to the response of different invertase preparations to the usual protein precipitation tests, are due to variations in the carbohydrate content of these preparations.

BETHESDA, MARYLAND RECEIVED MARCH 17, 1943

(21) (a) Willstätter, Schneider and Bamann, *Z. physiol. Chem.*, **147**, 248 (1925); (b) Willstätter, Schneider and Wenzel, *ibid.*, **151**, 1 (1926).

(22) Willstätter (ref. 21b) has reported a preparation with a time value of 0.084 minute, based upon the questionable procedure of determining the activity before dialysis and the solids in the less active material remaining after dialysis. Albers and Meyer [*Z. physiol. Chem.*, **228**, 122 (1934)] have reported a time value of 0.068 minute for a preparation which lost 90% of its activity as the result of six hours of dialysis. They have also reported two other preparations each with a time value of 0.088 minute. No statement was made with regard to the stability of these preparations to dialysis but the preparations were apparently unstable in aqueous solution.

(23) This appears to be due to variations, not only in the behavior of yeast from different sources, but also in the properties of kaolin (used as an adsorbent for invertase) from different sources.