method of preparing the intermediate nitrocresol.[11] The substituted phenylpyruvic acid was then converted to XIV *via* the sodium derivative by the method of Snyder and Williams.[12]

Catalytic Reduction of XIV to 5-Hydroxyindole (XV).—Recrystallized XIV (1.000 g.) was dissolved in 83 ml. of ethyl acetate and treated with hydrogen at 40 p.s.i. for 2 hr. at 80° in the presence of 1.64 g. of 25% palladium-on-carbon catalyst. The hot solution was filtered and concentrated to dryness. The colored material was purified by sublimation at 0.1 mm., and produced 0.374 g. (75%) of material melting at 103–107° (cor., Kofler). The infrared spectrum in Nujol, and the ultraviolet spectrum in ethanol corresponded with those published by Stoll, *et al.*,[9] for 5-hydroxyindole. Recrystallization of the sublimed product from benzene gave material which melted at 107.5–108° (cor., Kofler).

***o*-Nitromandelonitrile Benzoate (XVII).**—*o*-Nitromandelonitrile (XVI) was prepared from *o*-nitrobenzaldehyde *via* the bisulfite addition compound by the method of Reissert and Hessert.[13] The benzoate XVII was prepared from XVI by the method of Heller.[14]

Catalytic Reduction of XVII to Indole IX.—*o*-Nitromandelonitrile benzoate (XVII, 1.88 g.), 0.8 ml. of triethylamine, 1.0 g. of 30% palladium-on-carbon catalyst and 2.5 g. of anhydrous magnesium sulfate were washed into a hydrogenation bottle with 25 ml. of anhydrous ether. The vessel was flushed and pressurized to 45.0 p.s.i. with hydrogen gas. Most of the uptake, corresponding to 3.7 moles of hydrogen per mole of XVII, occurred in the first 1.5 min. of agitation. The vessel was vented and recharged four times at convenient periods during the course of the reaction. After 25.5 hr., the reaction was terminated, and the ether solution was filtered. The filtrate was extracted with two 5-ml. portions of 3% aqueous sodium hydroxide solution, two 2.5 ml. portions of water, two 5-ml. portions of 3% aqueous hydrochloric acid and two 2.5-ml. portions of water. The residual ether solution was dried over anhydrous magnesium sulfate and filtered, and the solvent was evaporated. A white crystalline material formed which was sublimed, producing 0.47 g. of glistening white plates melting at 51–52.5°. A mixture of the product with authentic indole melted at 51–52.7°, and the infrared spectra of the product and authentic indole were identical. The yield was 60%.

Attempted Catalytic Reduction of 2-Amino-3,6-dicarbethoxyindole (II) to 3,6-Dicarbethoxyindole (III).—A solution of 2.76 g. of II in 60 ml. of ethyl acetate was shaken with 1.0 g. of 30% palladium-on-carbon under 65 p.s.i. of hydrogen at 100–105° for 3 hr. When the bottle was opened, no ammonia odor was detected. The warm suspension was filtered, and the filtrate was replaced in the pressure bottle with 1.0 g. of fresh palladium-on-carbon catalyst. The mixture was treated with hydrogen under the above conditions for 4 hr. When the bottle was opened, no ammonia odor was detected. The catalyst was removed by filtration, and the filtrate was evaporated to dryness. The residue was II.

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URBANA, ILL.

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(13) A. Reissert and K. Hessert, *Ber.*, **57**, 964 (1924).
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[CONTRIBUTION FROM THE PIONEERING RESEARCH DIVISION, QUARTERMASTER RESEARCH AND ENGINEERING CENTER]

Enzyme Action on Partition Chromatographic Columns

BY ELWYN T. REESE AND MARY MANDELS

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This paper describes a method of obtaining an essentially continuous enzyme reaction on a two-phase column utilizing partition chromatography. In this method the enzyme, dissolved in an aqueous phase, is retained as the stationary phase on a column of hydrophilic solid such as cellulose. The substrate, moving through the column in the solvent phase, diffuses into the stationary aqueous phase where reaction with the enzyme occurs. Products of the reaction then diffuse back into the mobile phase and pass down the column. We have successfully used this principle for the reaction of β-glucosidase on several β-glucosides and invertase on sucrose. In some cases products have been obtained which result from the transfer of glycosyl groups to the alcohols used as the mobile phase. The enzymes used remain active under the conditions described for a number of weeks. While only hydrolysis and transfer reactions are reported here, it is believed that the principle involved is applicable to a wide variety of systems.

Introduction

Our original aim in using enzymes on columns was to devise a method for obtaining intermediates in enzyme reactions. In early work using adsorbed enzymes it was found that only a very small portion of the activity was retained. Such a procedure has been granted a patent (Stone)[1] but the claims indicate a slow and inefficient process. Using a two-phase column it was found that the enzyme remains free in the aqueous phase and retains high activity. This method approaches an ideal system with new substrate constantly entering from the solvent phase, and products being constantly removed. The enzyme action is continuous. There is neither inhibition by products, nor reduction in rate due to depletion of substrate. Further advantages are the absence of enzyme in the reaction products, the ability to obtain short-lived intermediates; the possibility of accomplishing a series of reactions by placing various enzymes at different levels in the column, and the ability to obtain synthetic products by transfer reactions. The process thus appears to have wider applicability than originally envisioned.

(1) I. Stone, U. S. Patent 2,717,852 (1955).

Methods

A. Preparation of Column.—The aqueous phase and the solvent phase were shaken together and allowed to stand overnight so as to become mutually saturated. The hydrophilic substance, cellulose (Solka floc, Brown Co.), was prepared as a slurry in the aqueous phase (0.01 *M* citrate *p*H 5.4) and dropped into the solvent phase to form a column (80 × 15 mm.). The column was then washed with the

solvent phase. Solvents were alcohols, usually 1-butanol.

B. **Enzyme.**—Two enzymes, β-glucosidase and invertase, were used in these experiments. After preparation of the column 1.0 ml. of aqueous enzyme solution was carefully introduced at the top of the column. The column was again washed with the solvent phase. The β-glucosidase was a lyophilized culture filtrate of *Aspergillus luchuensis* QM 873 containing 56 salicinase units per ml. One salicinase unit produces 4.0 mg. of glucose from 15 mg. of salicin in 10 ml. of citrate buffer at *p*H 5.4 in 1 hr. at 50°.

The invertase (Nutritional Biochemicals Company) contained 1430 units per ml. One invertase unit is the amount of enzyme causing 50% hydrolysis of 2 ml. of 0.10% sucrose, *p*H 5.4, in 30 minutes at 40°.

C. **Substrate.**—The substrates, usually glycosides, were dissolved in the alcoholic solvent phase in a concentration of 1.5 to 2.0 mg./ml., and about 50 ml. of the solution was passed through the column at a rate of 20 ml./hr. at room temperature. Columns were then washed with 150 to 250 ml. of solvent until neither products nor unreacted substrate were found in the eluate.

D. **Products.**—Ten-ml. aliquots from the column were dried on a steam-bath and the solids taken up in 5 ml. of water. These samples were analyzed for products of hydrolysis, glucose by the dinitrosalicylic acid method of Sumner and Somers,[2] saligenin by the Folin method of Lowry, *et al.*,[3] and *p*-nitrophenol by a modification of the *o*-nitrophenol method of Conchie.[4] Glucose was determined in the presence of cellobiose or fructose by the use of glucose oxidase. The amount of aglucone (saligenin, *p*-nitrophenol) is a true measure of the amount of substrate hydrolyzed. Where glucose found is less than equivalent to the aglucone, transglucosylation is indicated.

Total glucosides present in the eluates were determined by enzyme hydrolysis. The increase in glucose indicates the sum total of unreacted substrate and any new glucoside formed as a result of transfer of a glucosyl group to the alcohol used in the solvent phase. The increase in aglucone is a measure of the unreacted substrate only.

After analysis, the residual material from the eluates was concentrated and spotted on paper chromatograms to aid in characterization of the products. Sheets were developed in isopropyl alcohol:glacial acetic acid:water (67:10:23). Periodate benzidine was used to locate the glucosides, benzidine (Horrocks),[5] the reducing sugars and resorcinol–HCl[6] (Forsythe), the fructosides. Glycosides were further characterized by their susceptibility to hydrolysis by the appropriate enzyme. Saligenin was located with the phenol spray of Barton, *et al.*[7] Unreacted salicin was hydrolyzed by spraying the sheets with 0.1 *N* HCl and heating. The saligenin released was detected with the above phenol spray.

Results

A. β-Glucosidase.—β-Glucosidases attack β-glucosides by transfer of the glucosyl group to water or to an alcohol.

1. **Salicin.**—

$$\text{Salicin} + \text{HOH} \xrightarrow{\text{Enzyme}} \text{Saligenin} + \text{glucose}$$

$$\text{Salicin} + n\text{-butanol} \xrightarrow{\text{Enzyme}} \text{Saligenin} + n\text{-butyl } \beta\text{-glucoside}$$

Salicin (90 mg. in 45 ml. of 1-butanol) was passed through a column containing β-glucosidase in 7 hr. at room temperature, and 74% was split by the enzyme. Of the glucosyl transferred, 24% formed glucose and 76% formed a glucoside, probably *n*-butyl β-glucoside. In our solvent system, the latter has an R_G ($= R_{glucose}$) value of about 2.0 (Salicin $R_G = 1.72$).

In similar tests, other alcohols were substituted for 1-butanol. With isobutyl alcohol, 51% of the salicin was split by the enzyme in 6 hr. with *sec*-butyl alcohol, 33% in 7 hr.; and with *t*-amyl alcohol, 61% in 24 hr. With isobutyl alcohol as solvent, 67% of the glucosyl transferred went to form a glucoside, probably isobutyl β-glucoside. There was no evidence of transfer of glucosyl to the secondary or tertiary alcohols.

Since the reaction occurs at the top of the column, the products have ample time for separation as they pass through the rest of the column. Saligenin being more soluble in the solvent, moves more rapidly than glucose.

The times given in these results are those required for complete elution of the products from the column. Actual reaction time in the enzyme-containing layer is, of course, much shorter.

2. **Cellobiose.**—Cellobiose (109 mg.) in 1-butanol was passed through a column containing β-glucosidase. Eighty-three per cent. was split in 48 hr. About 77% of the transferred glucosyl was used to form a new glucoside (again probably *n*-butyl β-glucoside). The per cent. of glucosyl transferred to 1-butanol was the same for both salicin and cellobiose. This may be a function of the concentration of butanol (8–10%) in the aqueous phase where the reaction takes place.

3. **Methyl β-Glucoside.**—Glucose and an unknown β-glucoside, probably isobutyl β-glucoside ($R_G = 2.47$) were produced when methyl β-glucoside (R_G 1.68) in isobutyl alcohol was passed through a column containing β-glucosidase.

4. ***p*-Nitrophenyl β-Glucoside.**—*p*-Nitrophenyl β-glucoside (100 mg.) in isobutyl alcohol was 40% hydrolyzed on a similar column. Forty-two per cent. of the glucosyl transferred went to the alcohol.

5. **Glucose** (129 mg. in 1-butanol) was put through a similar column. Ninety-one per cent. of it was recovered in the eluates. No transfer reaction was anticipated and there was no evidence that any had occurred.

B. **Invertase.**—This invertase (yeast) transfers fructosyl groups from sucrose to water or to an alcohol and is therefore a β-fructofuranosidase.

$$\text{Sucrose} + \text{HOH} \xrightarrow{\text{Enzyme}} \text{Glucose} + \text{Fructose}$$

$$\text{Sucrose} + n\text{-butanol} \xrightarrow{\text{Enzyme}} \text{Glucose} + n\text{-butyl } \beta\text{-fructoside}$$

Invertase was added to the top of a column and followed by 65 mg. of sucrose in 1-butanol. All of the substrate was split in 2.0 hr. There was no evidence of glucosyl transfer to butanol. A fructoside, probably *n*-butyl β-fructoside, was detected on the chromatogram by its reaction with resorcinol–HCl. It accounts for about 5% of the fructose and has an $R_{fructose} = 1.90$. (The $R_{fructose}$ of sucrose = 0.74.)

C. **Other Tests.**—The question arose as to whether the enzyme is acting in a partitioning system or in an adsorbed condition. In our preliminary tests it was found that adsorbed enzymes had no more than traces of activity. To resolve

(2) J. B. Sumner and G. F. Somers, "Laboratory Experiments in Biological Chemistry," Academic Press, Inc., New York, N. Y., 1944.

(3) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(4) J. Conchie, *Biochem. J.*, **58**, 552 (1954).

(5) R. H. Horrocks, *Nature*, **164**, 444 (1949).

(6) W. G. C. Forsythe, *ibid.*, **161**, 239 (1948).

(7) G. M. Barton, R. S. Evans and J. A. F. Gardner, *ibid.*, **170**, 249 (1952).

this problem, invertase was added to a cellulose column and washed with the *aqueous* phase only. All the invertase quickly passed through the column. Enzyme adsorption is *not* playing an important role in these tests.

Since the enzyme is not adsorbed, the role of the solid phase in the column must be mechanical; to hold the aqueous phase in position and to maintain a maximum interfacial surface. To demonstrate this[8] the cellulose was eliminated by using a U-tube with a coarse sintered-glass filter at the base of one arm. This arm was partly filled with small glass beads and 4 ml. of invertase in the aqueous phase was placed in it. To the other arm was added sucrose in 1-butanol. A head of solvent was maintained so that 15–60 ml. of solvent per hour passed through the filter and rose through the aqueous phase as fine droplets, then overflowed into a collector. Under these conditions the sucrose (2 mg./ml. of solvent) was hydrolyzed completely with the formation of the same products as were produced on the column.

To test whether enzymes remain stable under such drastic conditions, an invertase column was set up. Sucrose, 3 mg. per ml. in 1-butanol, was passed through the column continuously for 28 days. The flow rate averaged 11 ml. per hour. Aliquots were collected daily and the per cent. of hydrolysis of the sucrose was determined.

While there was considerable day to day variation, probably due to fluctuations in the flow rate and in room temperature, there was no significant decrease in rate of hydrolysis over this period.

TABLE I

HYDROLYSIS OF SUCROSE BY INVERTASE

Day	% Hydrolysis	Day	% Hydrolysis	Day	% Hydrolysis
1	86	10	53	21	45
2	69	11	54	22	48
3	59	14	76	23	74
4	63	16	70	24	92
7	65	17	65	25	76
8	62	18	62	28	87
9	67				

(8) This modification was suggested by our colleague, Dr. J. D. Loconti.

Discussion

Enzymes are capable of acting continuously in a column operating on the principles of partition chromatography. We believe that the method is applicable to a wide range of enzymes and substrates and perhaps even to catalysts other than enzymes. The necessary details must be worked out for each system. One major problem is the selection of a solvent. Investigations on counter current distribution may suggest suitable solvents. Substrate and products should be reasonably soluble in both phases. The solvent used must not react with either substrate or products, except in certain types of transfer reactions. The enzyme must be reasonably stable under the conditions which exist on the column.

The fact that water quickly elutes the enzyme from the column shows that the enzyme is not adsorbed but exists free in the aqueous phase. As a result, the procedure is not limited to the use of enzymes in columns but may be used in any system involving a partitioning effect, such as the Craig countercurrent distribution apparatus, or any system involving continuous solvent extraction of an aqueous phase.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

The Effect of Ascorbic Acid on the Inactivation of Tyrosinase[1]

BY WALTER SCHARF AND CHARLES R. DAWSON

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Reports that the inactivation of the enzyme tyrosinase, during the aerobic oxidation of catechol, is reversed by ascorbic acid have been investigated and found to be illusory. It has been found that deceptive results may arise during colorimetric measurements of the reaction rate from (a) neglecting to keep the reaction-mixture continuously supplied with oxygen and (b) overlooking the fact that the initial oxidation product, *o*-benzoquinone, whose absorbance at 390 mμ forms the basis of the colorimetric method, is very unstable in aqueous solution. Experiments conducted under adequate conditions of oxygen supply lead to the conclusions that (a) inactivation of tyrosinase in the reaction-mixture is not caused by a product reducible by ascorbic acid, and so is not reversible by the latter, and (b) ascorbic acid, in the concentrations used for activity measurements by the chronometric method, has no activating effect on the enzyme.

The copper-enzyme, tyrosinase (polyphenol oxidase), of the mushroom suffers early and progressive inactivation during its catalytic participation in the oxidation of catechol to *o*-benzoquinone by molecular oxygen. The phenomenon has been the subject of much speculation and research,[2-5] as it is distinct in character from the more general type of enzyme inactivation due to environmental factors. As the result of experiments in these laboratories several years ago, Ludwig and Nelson,[2] and Miller, *et al.*,[6] reported that ascorbic acid has no effect on the activity of the enzyme. Consequently the acid has been widely used as a reducing agent in the reaction-mixture during catecholase activity measurements by the chronometric methods.[6,7] This innocuous role for ascorbic acid

(1) From a dissertation submitted by Walter Scharf in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University, 1957.

(2) B. Ludwig and J. M. Nelson, THIS JOURNAL, **61**, 2601 (1939).

(3) L. J. Roth, Dissertation, Columbia University, 1944.

(4) I. Asimov and C. R. Dawson, THIS JOURNAL, **72**, 820 (1950).

(5) L. L. Ingraham, J. Corse and B. Makower, *ibid.*, **74**, 2623 (1952).

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(7) W. H. Miller and C. R. Dawson, *ibid.*, **63**, 3375 (1941).