preformed amino acids. These postulations are being investigated.

*Summary.* 1) *A. niger* growth was prevented when DNB at a final concentration of $2.5 \times 10^{-5}$ M was added to the culture medium at any time up to 30 hours' incubation. When DNB was added at any time later, some additional growth occurred. The DNB effect appeared to be limited to molds and was static rather than cidal. 2) Growth inhibition by DNB was overcome by synthetic mixtures of amino acids, but not by ammonium nitrogen, vitamins or minerals. 3) Nitroaryl compounds can be reduced to relatively nontoxic arylamines by a sulfhydryl-dependent, pyridine nucleotide-linked enzyme from *A. niger*. 4) The possible significance of these findings is discussed.

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## Purification of Fungal Phenolsulfatase.* (24409)

RICHARD L. YOUNG† (Introduced by Edward A. Doisy)
*Dept. of Biochemistry, St. Louis University School of Medicine, St. Louis, Mo.*

The female sex hormones occur in urine not only in free form but also as glucuronides and sulfates which are hydrolyzed to the free compounds before quantitative measurement. Due to production of artifacts by acid hydrolysis, milder methods of enzymatic hydrolysis have been introduced. The sulfates are hydrolyzed by phenolsulfatase which was discovered by Neuberg and Kurono(1). Distribution and properties of the enzyme have been reviewed by Fromageot(2,3). After Abbott(4) pointed out that the most active fungal source of phenolsulfatase is Mylase P,‡ Cohen and Bates(5,6) used this commercial product for hydrolysis of urinary estrogen sulfates. Subsequently Katzman and associates (7) found that this preparation would hydrolyze estrone sulfate quantitatively. Since Mylase P is a mixture of fungal enzymes and no intensive purification of fungal phenolsulfatase has been reported, we have undertaken the preparation of this enzyme from Mylase P. In these studies fractionations with ethanol, zone electrophoresis on cellulose, and treatment with aluminum hydroxide gel were used to purify the enzyme.

*Materials and methods. Starting material.* The same lot of Mylase P was used during these studies. Its specific activity remained unchanged upon storage for one year at 5°C. Analysis for protein (Lowry *et al.*, 8) gave 12%, reducing sugars expressed as lactose(9, 10) 42%. *Determination of phenolsulfatase.* The procedure of Huggins and Smith(11) was used for determination of the activity of the enzyme. Potassium p-nitrophenylsulfate was synthesized by a modification of the procedure of Burkhardt and Wood(12). The substrate (0.005 M) in a solution of 0.1 M sodium acetate to which a few drops of $CHCl_3$

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† Walter Karr Fellow under auspices of Smith, Kline & French Labs., Philadelphia, Pa., 1951-1956. Present address, Biochemistry Section, Smith, Kline & French Labs., Philadelphia, Pa.

‡ Wallerstein Co., N. Y.

had been added was stable for as long as 7 months at 5°C. A solution of 3 ml of 0.5 M acetate buffer pH 5.8, 1 ml of solution of the enzyme, and 1 ml of solution of the substrate was incubated for 30 minutes at 37.5°C. Reaction was stopped by addition of 5 ml of 1.0 N NaOH solution. The absorbance of the basic solution was measured at 420 mμ, and corrected for absorbance of an appropriate blank containing only reagents. The release of 20 μg or less of p-nitrophenol resulted in a direct relationship between concentration of the enzyme and liberated p-nitrophenol. For convenience during this work, that amount of enzyme which would release 10 μg of p-nitrophenol in 30 minutes was designated as 1 unit of phenolsulfatase activity. *Index of purity.* Specific activity (S.A.) is the number of units of enzyme per mg of protein. *Purification procedures.* All purification procedures except fractionations with ethanol were performed in a cold room at 5°C. *Extraction of Mylase P.* Phenolsulfatase was brought into solution by extracting 1 weight (g) of Mylase P with 2.5 volumes (ml) of distilled water for 1 hour. The insoluble residue was collected by centrifugation at *830 x g* for 10 minutes and washed twice with small volumes of distilled water. Supernatant and wash solutions were combined and diluted with distilled water to give a 3% concentration of protein (pH 5.5-6.0). *Fractionations with ethanol.* Solutions of the enzyme were transferred to a cold room (–15°C); an equal volume of 47.5% ethanol (–15°C) was added according to the general procedure of Cohn *et al.*(13). Then 95% ethanol (–15°C) was added in order to attain the final concentration of ethanol. All concentrations of ethanol are reported in per cent by volume. Inert material was precipitated from the aqueous extract Mylase P by addition of ethanol to a concentration of 38%. After ½ hour the precipitate was collected by suction filtration through Celite 545 filter aid§ in chilled (–15°C) apparatus and washed with small portions of chilled 38% ethanol. Filtrate and washings were combined and diluted with 38% ethanol to give a 1% concentration of protein. The solution was chilled and the enzyme precipitated by adjusting concentration of ethanol to 52%. After 1 hour the supernatant liquid was decanted and the precipitate collected by centrifugation in chilled (–15°C) cups at *830 x g* for 10 minutes. The precipitate was dissolved in 0.025 M acetate buffer pH 5.0 and concentration of protein adjusted to 3%. The enzyme was precipitated again by addition of ethanol to a concentration of 29%. After 1 hour the precipitate was collected by centrifugation at –15°C for ½ hour at *1,100 x g,* dissolved in 0.025 M acetate buffer pH 5.0, and adjusted to a concentration of protein of 1%. Phenolsulfatase of this solution was then precipitated in the same manner as the preceding precipitation at pH 5.0. The precipitate was dissolved in 0.025 M barbital buffer pH 8.5 and dialyzed against the same buffer overnight. The impermeate was diluted with this buffer to the desired concentration of protein and used for electrophoresis on blocks of cellulose. *Paper electrophoresis.* 0.05 ml of a buffered solution of the enzyme containing 2.2 to 4.8 mg of protein were applied to the center of a 57 x 11.5 cm strip of Whatman 3 MM filter paper mounted in a plastic paper electrophoresis apparatus.‖ Electrophoresis was then conducted under the desired conditions. At termination of migration the paper was cut into 1 cm sections, and each was eluted with distilled water. Clear supernatant solutions obtained after centrifugation of the suspended filter paper were used for determination of enzyme and protein. *Electrophoresis on blocks of cellulose.* Each block of cellulose was prepared from 200 g of Whatman Standard Grade B Quality Cellulose Powder for Chromatography which had been washed with 0.025 M barbital buffer pH 8.5, resuspended in this buffer, and placed in a 40 x 8 x 1 cm plastic tray. Five blocks were used simultaneously for the product obtained from 300 g of Mylase P. The trays were connected with the buffer compartments by 12 x 8 cm strips of Whatman No. 1 filter paper. Then 7 ml of the above solution of the enzyme in 0.025 M barbital buffer pH 8.5 and containing 200 mg of protein were incorporated into each of the

§ Johns Manville Co., N. Y.

‖ Paper Electrophoresis Apparatus Model 1400, Research Specialties Co., Berkeley, Calif.

TABLE I. Preparation of Partially Purified Phenolsulfatase.

| Fraction | Recovery | | | |
|---|---|---|---|---|
| | Total mg protein | Total activity of enzyme | Specific activity | % of original enzyme |
| Mylase P | 36,000 | 210,000 | 5.8 | 100 |
| Aqueous extract | 29,180 | 202,000 | 6.9 | 96 |
| First ethanol ppt. | 10,150 | 176,400 | 17 | 84 |
| Second " " (pH 5.0) | 3,300 | 134,500 | 41 | 64 |
| Third " " ( " ) | 1,900 | 119,800 | 63 | 57 |
| Impermeate from dialysis of barbital solution (pH 8.5) of third ethanol ppt. | 1,600 | 119,800 | 75 | 57 |
| Combined eluates from electrophoresis on blocks of cellulose | 600 | 92,500 | 154 | 44 |
| Eluates from $Al(OH)_3$ gel | 110 | 51,200 | 465 | 24 |
| Impermeate from dialysis of ethanol ppt. from eluates of $Al(OH)_3$ gel | 84 | 45,700 | 544 | 22 |
| Lyophilized product | 65 | 26,600 | 410 | 13 |

central and –15 cm sections of the blocks according to the procedure of Kunkel and Slater (14). Electrophoresis was allowed to proceed for 18 hours at 12.5 v cm and 9-11 ma. Then 1 cm segments of the blocks were removed and eluted with distilled water. The cellulose was collected by centrifugation and the clear supernatant solutions were used for determination of enzyme and protein. Those fractions having a specific activity greater than the product subjected to electrophoresis were combined for further purification by adsorption. *Adsorption on aluminum hydroxide gel.* $Al(OH)_3$ gel was prepared according to the procedure of Marshall and Welker(15). Aliquots of the suspended gel were dried to constant weight at 110°C, weight 17 mg/ml. Combined eluates from the blocks of cellulose were diluted to 3 liters with distilled water (final pH 8.0), adjusted to pH 5.4 by the addition of 60 ml of 0.1 M acetate buffer pH 5.0, and adsorbed on 0.4 ml of gel (dry weight 6.8 mg) per 10 mg of protein for 15 minutes. The gel was collected by centrifugation and washed twice with small portions of distilled water. Foreign protein was eluted by resuspending the gel in 2 ml of M/90 Sorensen phosphate buffer pH 6.6(16) per 10 mg of adsorbed protein 2 times followed by washing once with a small volume of distilled water. Supernatant and wash solutions were discarded. Phenolsulfatase was eluted from the gel with 1.5 ml of 0.1 M $K_2HPO_4$ solution per 10 mg of adsorbed protein; the elution was repeated twice. The eluates were combined, frozen, and kept overnight at –15°C. The solution was thawed and the enzyme precipitated by ethanol at a final concentration of 59%. After 1 hour the precipitate was collected by centrifugation at –15°C for ½ hour at *1,100 x g*, dissolved in a minimum volume of distilled water, and dialyzed against distilled water for 4 hours. *Lyophilization.* Lyophilization was conducted in the conventional manner while the sample of the enzyme was immersed in a bath of salt and ice.

*Results.* The results of application of the above methods are given in Table I. During the work all fractions including those which were discarded were analyzed for phenolsulfatase. Total activity at each step showed that no significant destruction of the enzyme had occurred. Phenolsulfatase was completely extracted from Mylase P with distilled water. During the process 26% of the inert solids of the preparation were eliminated. The extract, however, contained practically all of the initial protein (S.A. 6.9). Subsequent fractionations of the enzyme with ethanol resulted in a product which was suitable for electrophoresis on blocks of cellulose (S.A. 63). A similar product which was dialyzed briefly against distilled water contained less than 1% of the reducing sugars and 11% of the solids of Mylase P.

Pilot paper electrophoresis experiments were performed with the enzyme to determine the most suitable conditions for purification on a large scale. During these studies the enzyme in 0.025 M acetate buffer migrated

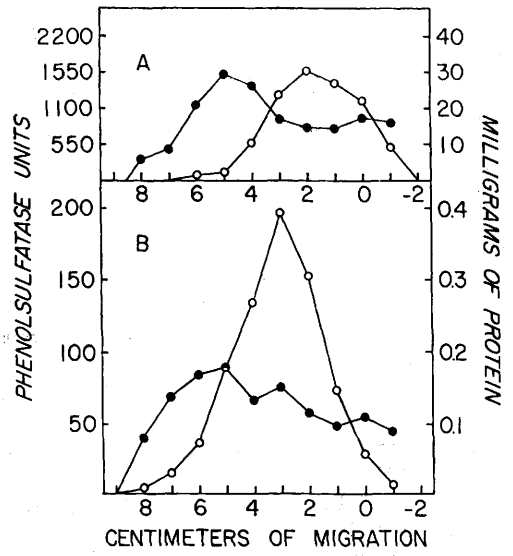


FIG. 1. Distribution of enzyme (○) and protein (●) after electrophoresis. Distances from origin (○) are indicated on abscissae in cm of migration toward the anode or –cm toward the cathode. Typical results after preparative electrophoresis of phenolsulfatase (S.A. 63) on blocks of cellulose powder for 18 hours at 12.5 v/cm and 9-11 ma in presence of 0.025 M barbital buffer pH 8.5 at 5°C are shown in Fig. 1A. (Distribution of the product applied at –15 cm is not shown.) Fig. 1B shows results after electrophoresis of enzyme (S.A. 410) on filter paper for 20 hours at 5 v/cm and 2-3 ma in presence of 0.02 M potassium phosphate buffer pH 6.7 at 5°C.

toward the anode at pH 5.5 and toward the cathode at pH 4.5. These data suggest that the isoelectric point of phenolsulfatase is in the vicinity of pH 5.0, but no corrections were made for electroendosmosis. Recovery of enzyme was 83-97%; of protein 88-109%. The most satisfactory purifications on paper strips were achieved in the presence of 0.025 M barbital buffer pH 8.5.

Typical results for electrophoresis on blocks of cellulose are shown in Fig. 1A. Results were similar for the product introduced at –15 cm and from block to block. Total recovery of enzyme averaged 92%, and those fractions having specific activities greater than the product subjected to electrophoresis contained 78% of the enzyme. A brown pigment moved 10 cm toward the anode resulting in extensive decolorization of phenolsulfatase.

Aluminum hydroxide gel was chosen for adsorption of phenolsulfatase because it was found to adsorb more enzyme than protein at pH 4.2-6.1, and more protein than enzyme was eluted from the gel with dilute phosphate buffers at pH 6.2-6.8. The enzyme was eluted from the gel with 0.1 M $K_2HPO_4$ solution immediately since storage of phenolsulfatase in the absorbed state resulted in considerable loss of its activity.

The product obtained from the sequence of purification summarized in Table I showed a specific activity of 544 and a recovery of 22% of the original enzyme. Since earlier experiments showed that phenolsulfatase could be lyophilized without loss of activity if the solution of the enzyme was immersed in a bath of salt and ice, the product was dried in that manner. Considerable inactivation occurred during lyophilization; however, the resultant product (S.A. 410) weighing 127 mg was stored under desiccation in the dark at 5°C. A small portion of the product which was reserved for study of its stability lost no activity during storage for 11 days, but 22% inactivation occurred within 9 weeks.

Two samples of the dried product were subjected to electrophoresis on paper strips for 20 and 31 hours, respectively, in the presence of 0.02 M potassium phosphate buffer pH 6.7. The voltage gradient was 5 v/cm, and the current 2-3 ma. Specific activities of the fractions containing maximum enzymatic activity were 1310 and 1360, respectively. In both experiments the distribution of protein exhibited 3 maxima whereas the enzyme showed only a single maximum. The sum of the phenolsulfatase of those fractions analyzed for the enzyme was 86% of the product applied to the paper. Results after migration for 20 hours are shown in Fig. 1B. The eluate containing enzyme (S.A. 1310) was stable to freezing overnight at –15°C, but refreezing and storage for 6 days at –15°C resulted in 23% inactivation.

Owing to the marked variation in composition of the starting material, some difficulty was encountered with products of lower concentration of enzyme. Three lots of Mylase P received from the manufacturer contained only half as much phenolsulfatase activity as the material used during these studies. Attempts to prepare enzyme of high activity (S.A. 544) from the less active starting ma-

terial, and to avoid the loss incurred during lyophilization by immediately subjecting the product to electrophoresis on blocks of cellulose were not successful in the limited time for exploration of these points.

*Discussion.* Although the activities of the final products were approximately 230 times that of Mylase P on the basis of purification of protein, the activities were approximately 1900 times that of Mylase P on a gravimetric basis since the starting material contained only 12% protein. In spite of these extensive purifications, the results indicate that fungal phenolsulfatase was not pure by the criterion of paper electrophoresis at pH 6.7. The retention of most of the enzymatic activity during electrophoresis, however, suggests that electrophoresis on blocks of cellulose might be suitable for further purification of larger amounts of the enzyme.

*Summary.* Fungal phenolsulfatase of Mylase P was purified by fractionations with ethanol, zone electrophoresis on cellulose, and treatment with aluminum hydroxide gel. Related to the activity of the original Mylase P the enzyme was purified approximately 1900 times. On the basis of purification of the protein of Mylase P approximately 230-fold purifications were achieved.

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## Biosynthesis of Cholesterol in Animal Blood.* (24410)

V. S. RAUT, C. A. FISH AND GREGORY PINCUS
*Worcester Foundation for Experimental Biology, Shrewsbury, Mass.*

Investigations were carried out on biosynthesis of cholesterol from $C^{14}$ labelled sodium acetate, in human leukemic blood, and an apparently high rate of synthesis was observed in blood from patients suffering from myelogenous leukemia (unpublished data). Keeping this in mind, an attempt was made to find a good source of the enzyme system responsible for biosynthesis of cholesterol in blood. Hence, blood samples from different animals like rat, rabbit, pig, piglet, calf and cow were tried. Results with calf blood seem to be very encouraging.

*Materials and methods.* Blood samples from cows, calves and pigs were procured from local slaughter houses. Calf blood was taken from very young animals, 3 to 5 years old. Five calf blood samples were analyzed in 2 separate sets of 3 and 2 each. Adult cows and pigs, Sprague-Dawley strain adult male rats (300-400 g weight), and New Zealand strain adult male rabbits (6 lb weight) were used for their respective blood samples. Blood samples from rats, rabbits and piglets

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