false positive result is also slight since so far no enzyme is known, other than that type of penicillinase (penicillin β -lactamase) for which the test was devised, which attacks penicillin to yield a product reacting with iodine.

SUMMARY

1. A method is described for the chromatographic detection of penicillins and related compounds and of penicillinase based on the reaction of iodine with penicilloic acids.

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Purification of Latent Phenolase from Broad-Bean (*Vicia faba*) Leaves

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The paucity of information on the nature and function of the plant phenolases is apparent from a recent review (Bonner, 1957) on this family of oxidases. An interesting contribution has been the large-scale fractionation and purification of mushroom phenolase by Kertesz & Zito (1957). More recently reports have appeared of the chromatography on cellulose ion-exchange agents of phenolase from mushroom (Frieden & Ottesen, 1959), *Polyporus versicolor* (Malmström, Fähræus & Mosbach, 1958) and from mammalian tissue (Brown & Ward, 1958). A latent phenolase is present in the leaves of the broad bean, and Kenten (1957, 1958) has studied in some detail the activation of this enzyme in a partially purified preparation. During the course of work in these laboratories on the characterization of the proteins of the broad-bean leaf, various fractions, as judged by their colour, were obviously contaminated with Kenten's latent phenolase. This brief paper describes the chromatographic method used quite early in the work for the removal of the enzyme from pigments and from the major protein components of the leaf juice.

EXPERIMENTAL

Preparation of leaf extract. The broad-bean plants (Windsor Improved) were grown to various stages of maturity in a garden or in John Innes Compost in a heated greenhouse. The leaves (50–100 g. fresh weight) were

vacuum-infiltrated with a solution containing 0.03M-potassium ascorbate, 0.2 mM-sodium ethylenediamine-tetra-acetate and 0.03M-potassium phosphate buffer, pH 7.5. Excess of solution was then squeezed from the leaves before they were ground with the aid of a little sand in a pestle and mortar at 4°. The suspension was centrifuged at 20 000g for 45 min., and the supernatant dialysed against many changes of buffer containing 5 mM-ascorbate, 4 mM-phosphate and 4 mM-citrate, adjusted to pH 6.5 with potassium hydroxide. The latent phenolase solution was freeze-dried, and the residue was taken up in 2–3 ml. of water, centrifuged if necessary to clarify, and dialysed for 1 hr. against 5 mM-potassium phosphate buffer at pH 7.

Column chromatography. Diethylaminoethylcellulose (DEAE-cellulose) was prepared from Solka Floe SW-40B (Johnsen, Jorgensen and Wettre Ltd., London, E.C. 4) according to Peterson & Sober (1956) but with only half the quantity of the reagent α -chlorotriethylamine hydrochloride. The resulting adsorbent contained 0.50 m-equiv. of basic nitrogen/g. A column of DEAE-cellulose (30 cm. $\times$ 1 cm.) was prepared from a slurry in 5 mM-potassium phosphate buffer, pH 7, and packed down under a pressure of 8 lb./in.². The phenolase solution was transferred to the column and eluted rapidly with 5 mM-potassium phosphate buffer at pH 7. After 20 ml. had passed through the column, 0.5M-potassium hydroxide was applied through a 100 ml. mixing chamber to establish a concentration gradient and a pH gradient to 8.5. Duplicate samples of 0.1 ml. were taken from 2 ml. fractions for analysis by the Folin-Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) for protein and by β -(3:4-dihydroxyphenyl)-DL-alanine-Teepol for phenolase. The latter reagent was prepared from 5 ml. of Teepol (Anionic detergent, Shell Chemical Co.), 30 mg. of β -(3:4-dihydroxyphenyl)-DL-alanine and 0.05M-potassium phosphate at pH 6 to a volume of 50 ml. This reagent (0.5 ml.) and 4 ml. of buffer, pH 6, were added to each portion, and the extinction of the

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orange-coloured solution was read at 495 $m\mu$ after a period of 15–20 min. at room temperature. Although this method is not as accurate as measuring the rate of change of extinction for each enzyme concentration, it is quite adequate for monitoring the column effluent. A second column of DEAE-cellulose was operated at pH 7 with increasing ionic strength only.

Catecholase activity. Catecholase activity was estimated by the method described by Dawson & Magee (1955) which

involves measuring the production of *o*-benzoquinone as a function of time from a given amount of enzyme. A 'unit of activity' corresponds to *o*-benzoquinone production of 1.49×10^{-8} mole/sec.

RESULTS

A chromatogram of whole juice from broad-bean leaves on DEAE-cellulose is shown in Fig. 1. The bulk protein of the extract can be divided into a number of fractions completely free of phenolase activity, and although protein is eluted continuously only a small amount is associated with the enzyme. Most of the total protein is eluted before pH 8.5 is reached; increasing the ionic strength of the eluent without increasing the pH causes considerable overlap of the main protein peak with the phenolase activity. The pooled latent phenolase fractions were concentrated and the sample re-chromatographed on another column of DEAE-cellulose. A single and fairly symmetrical peak was obtained as shown in Fig. 2. The overall yield after passage through the two columns was greater than 90%. The chronometric method of Dawson & Magee (1955) seemed the most convenient way for assessing the specific activity of the enzyme with catechol as substrate. According to this method juice from broad-bean leaves had a specific activity, after activation with Teepol, of 4–5 units/mg. of nitrogen. Preparations obtained by chromatography on the first and second DEAE-cellulose columns had specific activities in the range 1800–2500 and 2000–3000 units/mg. of nitrogen respectively.

Unlike all active phenolases so far reported in the literature, activated broad-bean phenolase is strongly adsorbed on DEAE-cellulose, the semi-translucent appearance of the adsorbent changing during the process of adsorption to an opaque white. No chemical modification of the adsorbent appears to take place, the nitrogen content and titration curve remaining unchanged.

No further purification of the enzyme has been attempted beyond the above, but in view of its stability and the high yields obtainable it would seem to be an ideal member of the plant phenolases for structural investigation and for further metabolic studies.

SUMMARY

1. Latent phenolase from broad-bean leaves may be removed from pigments and the major protein components of the leaf juice in one operation by gradient elution from a column of diethylaminoethylcellulose.

2. Rechromatography of the enzyme on the same adsorbent yields a preparation of high catecholase activity in 90% overall yield.

Fig. 1. Chromatography of whole juice from 50 g. of fresh leaves on a column (30 cm. $\times$ 1 cm.) of DEAE-cellulose; 2 ml. fractions. —, Folin-Lowry method with 0.1 ml. portions, extinction measured at 570 $m\mu$; - - -, phenolase activity of 0.1 ml. portions in terms of coloured oxidation products formed in 15 min. from β -3:4-dihydroxyphenylalanine in the presence of Teepol, extinction measured at 495 $m\mu$. A, 5 mM-Phosphate buffer at pH 7.0; B, 0.5M-KOH through a 100 ml. mixing chamber to produce gradient to pH 8.5.

Fig. 2. Rechromatography of latent phenolase on a column (45 cm. $\times$ 1 cm.) of DEAE-cellulose. Analysis of 2 ml. fractions was as in Fig. 1. A, 5 mM-Phosphate buffer at pH 7.0; B, gradient change to 0.5M-phosphate at pH 7.0 through a 100 ml. mixing chamber. Specific activity of pooled peak was 2000–3000 units/mg. of nitrogen.

3. Unlike other known active phenolases the enzyme released from a broad-bean leaf extract by Teepol is adsorbed strongly on diethylamino-ethylcellulose.

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An Enzyme from Rat Liver Catalysing Conjugations with Glutathione

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Many compounds are excreted in urine and bile as mercapturic acids, which are thio ethers of acetylcysteine, and there has been considerable speculation on the source of the cysteine used for the biosynthesis of these compounds. Evidence that glutathione is involved was supplied by the work of Barnes, James & Wood (1959), who found that the levels of glutathione in the liver decreased when mercapturic acid-forming compounds were administered to rats. Furthermore, when *p*-chlorobenzyl chloride was given to a rabbit, investigations with paper chromatography provided evidence for the presence of *S*-(*p*-chlorobenzyl)-glutathione in the liver, and this compound could be converted into *S*-(*p*-chlorobenzyl)cysteine by liver slices and homogenates and by glutathionase (Bray, Franklin & James, 1959*a*). The *N*-acetylation of some of the *S*-substituted cysteines to the corresponding mercapturic acids was demonstrated *in vivo* and in tissue preparations *in vitro* (Bray, Franklin & James, 1959*b*).

It has been shown that rat-liver slices convert naphthalene into *S*-(1:2-dihydro-2-hydroxy-1-naphthyl)glutathione (Booth, Boyland & Sims, 1960*b*) and 1:2-dihydronaphthalene into *S*-(2-hydroxy-1:2:3:4-tetrahydro-1-naphthyl)glutathione. This latter reaction could also be accomplished by rat-liver microsomes in the presence of reduced triphosphopyridine nucleotide, oxygen and glutathione, and the amount of glutathione derivative formed was increased by the addition of the soluble

fraction of rat liver (Booth, Boyland, Sato & Sims, 1960*a*).

This paper describes the partial purification and some of the properties of the liver enzyme which catalysed the formation of glutathione derivatives from various compounds.

EXPERIMENTAL

Materials. Triphosphopyridine nucleotide (TPN) as the dihydrate of the sodium salt, reduced TPN (TPNH₂) as the sodium salt, diphosphopyridine nucleotide (DPN), reduced DPN (DPNH₂) as the disodium salt, glucose 6-phosphate as the disodium salt, flavin mononucleotide as the monosodium salt dihydrate, adenosine 5-triphosphate (ATP) as the disodium salt and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co. Pyrophosphate buffers were prepared from K₄P₂O₇ and Na₂H₂P₂O₇ and phosphate buffer from KH₂PO₄ and Na₂HPO₄. Concentrations of the constituents of the reaction mixtures refer to the final concentrations at the beginning of the incubations.

S-(2-Chloro-4-nitrophenyl)-L-cysteine. This was prepared from 2-chloro-4-nitroaniline by the method of Parke & Williams (1951), when it formed pale-yellow needles from aq. ethanol, m.p. 176–178° (decomp.) (Found: N, 10.15; Cl, 12.45. Calc. for C₆H₄O₄N₂ClS: N, 10.1; Cl, 12.8%). Bray *et al.* (1959*b*) report m.p. 182° for this compound prepared by the method of Suter (1895). Acetylation of the compound with acetic anhydride in 2*N*-NaOH yielded *N*-acetyl-*S*-(2-chloro-4-nitrophenyl)-L-cysteine in cream needles from ethanol, m.p. 193°. Bray, James & Thorpe (1957) report m.p. 192–194° for this derivative.